PROCESSING RICE BRAN TO YIELD ADDED-VALUE OIL BASED EXTRACTS

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Thesis Submitted to the University of Nottingham for the

Degree of Doctor of Philosophy

JULY 2012

ABSTRACT

Rice bran, a by-product from rice milling, is an excellent source of natural antioxidants. Lipids in rice bran appear as small spherical droplets called oil bodies. This work attempted to recover the oil bodies from rice bran (fresh, stored and heat-treated) and to determine their chemical, biochemical and physical properties *ex vivo*. As revealed by transmission electron microscopy, oil bodies were observed mainly in the sub-aleurone and aleurone layer of brown rice. Oil bodies were successfully recovered from rice bran and were enriched in tocochromanols and oryzanol (656 - 1,006 mg/kg lipid and 8,880 - 9,692 mg/kg lipid respectively). Further washing to remove extraneous protein and non-associated compounds, effective lipid concentration increased while protein concentration decreased. The washed oil body preparation contained approximately 35 - 68 % tocochromanols and 60 - 62 % oryzanol of the parent rice bran oil. Therefore, the majority of tocochromanols and oryzanol molecules appeared to be intrinsically associated with rice bran oil bodies *ex vivo*. Fatty acid composition of rice bran oil bodies was similar to that of parent rice bran. SDS-PAGE of proteins present in differentially washed oil body preparations revealed similar protein profiles; however, there was a relative enrichment of the bands at 16 - 18 kDa (typical molecular weight of oleosins). Rice bran oil bodies possessed negatively charged surface (-30 mV) at neutral pH. As the pH of the oil body suspension was lowered to the pH near pI (about pH 4 - 5), zeta potential of the oil bodies approached zero and the suspension had the least physical stability; aggregation and the least relative turbidity.

The biochemical instability of rice occurs immediately after milling, which leads to the limited use of rice bran for human consumption. Free fatty acids and lipid hydroperoxides in rice bran and corresponding oil bodies increased significantly ($P<0.05$) during storage. Oil bodies recovered from stored rice bran aggregated and coalesced. 41% of tocochromanols in the oil bodies had decomposed while the concentration of oryzanol was relatively stable during the storage. Rice bran heat treatments (pan roasting and extrusion) caused the coalescence of oil bodies *in vivo* and the instability of an oil body suspension *ex vivo*.

The main findings of this study were that rice bran oil bodies were enriched in phytochemicals including tocochromanols and oryzanol and were resistant to oxidation providing that the oil bodies were still intact. The oil bodies could delay the onset of lipid oxidation of stored lipids inside the oil bodies. This may be explained by the physical barrier of surface membrane protein (oleosin) against pro-oxidants and the intrinsic association between the oil bodies and phytochemicals in rice bran.

ADDITIONAL ACHEIVEMENTS

Award

- Biotechnology Division Student Award 2011 in recognizing outstanding student paper entitled “Tocochromanols and oryzanol - associated components of rice bran and rice bran oil bodies” presented at the AOCS Annual Meeting & Expo, Cincinnati, USA.

Article in Press

- Nantaprapa Nantiyakul, Samuel Furse, Ian Fisk, Gregory Tucker and David A. Gray. Phytochemical composition of *Oryza sativa* (Rice) bran oil bodies in crude and purified isolates. Journal of the American Oil Chemists’ Society, DOI: 10.1007/s11746-012-2078-y, Published online: 19 May 2012.

Articles in Preparation

- Nantaprapa Nantiyakul and David A. Gray. Impact of heat treatment and storage on the quality of *Oryza sativa* bran and its oil bodies.
- Nantaprapa Nantiyakul, Samuel Furse, Ian Fisk, Gregory Tucker and David A. Gray. The isolation of oil bodies from *Oryza sativa* bran and studies of their physical properties.

ACKNOWLEDGMENTS

I wish to express my profound gratitude towards my research supervisors Dr. David Gray and Prof. Greg Tucker for their helpful comments, inspiring suggestions and encouragement throughout the study. Without their supports, this study could not have been successfully accomplished. My sincere also goes to Dr. Mita Lad and Dr. Ian Fisk for guiding my experimental work during my first year study and Phil Glover and Dr. Guy Channel, who always generously provide technical guidance. Thanks to Dr. Avinash Kant and Kanchana Minson for their helps and suggestions to complete this dissertation. Also, I would like to thank all colleagues in Food Science Division especially those in lipid body group for their helps and support.

Sincere gratitude is conveyed to my dear parents for their endless love, support, encouragement and understanding from the day I was born. My appreciation also goes to Jaturapon, Srichan, and Thada who always there for me. Thank to all my Nottingham friends for enjoyable experience and wonderful time in UK.

Finally, financial support from the Agricultural Development Agency (ARDA), Thailand is gratefully acknowledged.

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ABBREVIATIONS

Alkali-COB	Alkali-Crude Oil Body
Alkali-WOB	Alkali-Washed Oil Body
ANOVA	Analysis of Variance
a_w	Water Activity
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
COB	Crude Oil Body
dwb	Dry Weight Basis
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
FAME	Fatty Acid Methyl Ester
FFA	Free Fatty Acid
FRAP	Ferric Reducing Antioxidant Potential
GAE	Gallic Acid Equivalent
GC/MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
MBB	Mini-Beadbeater
MP-COB	Mortar and Pestle Crude Oil Body
OB	Oil Body
PA	Phosphatidic acid
PC	Phosphatidylcholine
PDA	Photodiode Array

PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
pI	Isoelectric Point
PL	Phospholipid
RB	Rice Bran
RH	Relative Humidity
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SPME	Solid-Phase Microextraction
T	Tocopherol
T3	Tocotrienol
TAG	Triacylglycerol
TBARS	Thiobarbituric Reactive Substances
TEM	Transmission Electron Micrograph
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
TPTZ	2,4,6-Tripyridyl-1,3,5-triazine
UWOB	Urea-Washed Oil Body
WWOB	Water-Washed Oil Body

1. INTRODUCTION

Rice is a major cereal crop cultivated globally. Rice bran, a by-product from rice milling, is mainly used as animal feeds because of the rapid hydrolysis of oil into free fatty acids. The biochemical instability of rice bran occurs immediately after milling. However, it is an excellent source of inexpensive, high content bioactive compounds. Rice bran is rich in natural antioxidants including tocochromanols (tocopherols and tocotrienols) and oryzanol. Conventional rice bran oil extraction and refining alter the composition of the oil. A considerable amount of these phytochemicals is lost in the processes.

Lipids in rice bran appear as small spherical droplets called oil bodies. These organelles have been recovered from oilseeds and display surprising physical and chemical stability *ex vivo*. Very little work, however, has been done to characterize oil bodies from cereal grains. The work described in this dissertation attempted to recover the oil bodies from rice bran (fresh, stored and heat-treated) and to determine their chemical, biochemical and physical properties *ex vivo*. The underpinning idea for this work is that oil bodies could be recovered from rice bran at different stages of deterioration, and provide oxidatively-stable rice bran oil as a natural emulsion that is

rich in phytochemicals (tocochromanols and oryzanol) and low in free fatty acids.

1.1. Rice production

Rice is one of the staple foods for human consumption, especially in East and South East Asia, where it supports nearly half of the world's population. Total annual world productions of rice bran oil and rice paddy in 2007 were 0.83 and 523 million tonnes respectively. Annual world production of rice bran oil and rice paddy in 2007 is compared in Figure 1.1. While most countries in East and South East Asia were the world top rice paddy producers, only some countries such as India, China, Japan and Thailand produced rice bran oil.

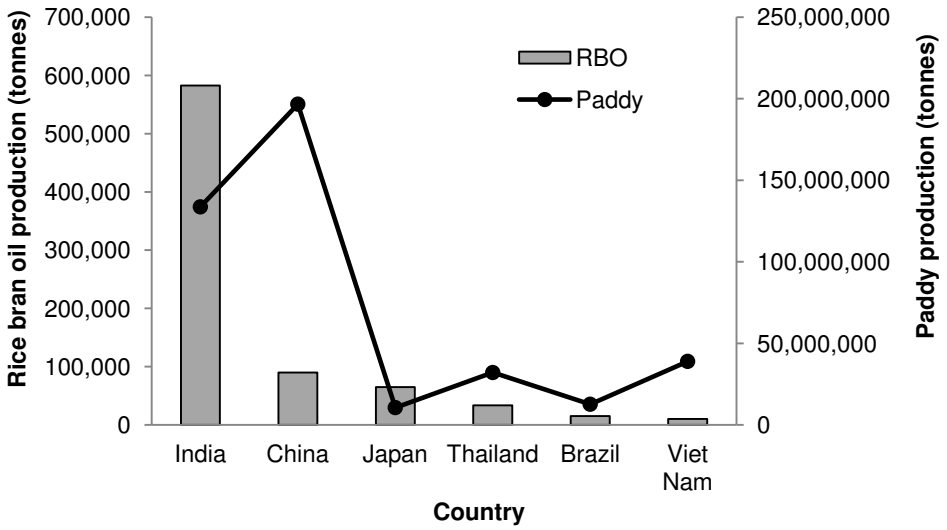


Figure 1.1 Comparison of annual world production of rice bran oil and rice paddy from different countries in 2007 (FAOSTAT, 2007)

Since rice bran oil can be extracted only from the large milling operations where stabilization systems are economically provided, only 11 % of the rice bran produced was used for rice bran oil extraction in 2007. Rice bran oil represented about 1 % of the total annual world vegetable oil production. Despite the very small production of rice bran oil relative to other common vegetable oils, it offers several unique properties that are very attractive for niche markets.

1.2. Rice structure

Rice (*Oryza sativa*) is a monocot which classified into Clade Commelinids, order *Poales* and family *Poaceae* (formerly *Gramineae* or grass) (Chase, 2003). Rice grain or rice kernel (Figure 1.2) composes of hull (20 %), bran and germ (10 %), and starchy endosperm (70 %) (Orthoefer, 1996).

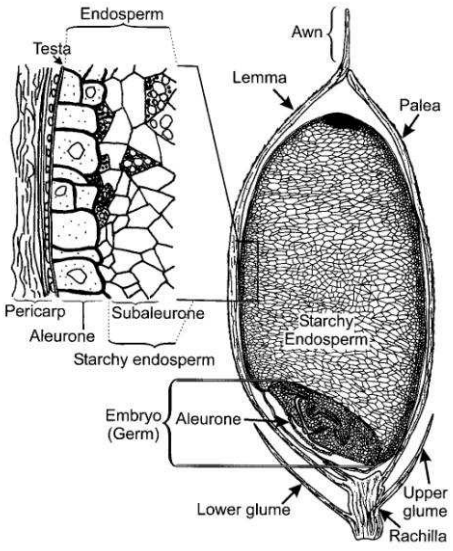


Figure 1.2 Rice kernel structure (Champagne, 2004)

1.3. Rice milling

Rice milling is a process which removes foreign materials, husk, bran and broken kernels from rice and produces the grain for various commercial purposes (Champagne, 2004). Rice milling operations are composed of five major stages including 1) cleaning of paddy, 2) removal of husks from rice kernels, 3) removal of bran layers from brown rice, 4) milled rice grading or removal of broken grains, and 5) blending and packaging of milled rice.

Paddy rice or rough rice is the rice harvested from the field. The rice kernels are still encased with protective husks. The paddy rice is fist cleaned to remove different types of impurities before introducing rice into the milling facility. Contaminants such as stones, mud balls, straw, metal, glass, grass, weed seeds and other grains may be present in the rice during harvesting and storage.

Afterwards, the husk is removed from the rice kernel to produce brown rice. A paddy husker is used to remove the husk by feeding rice through a small gap between two rubber rolls turning at different speeds in opposite directions. The husk is sheared from the brown rice while the rice paddy attempts to travel at the same speed corresponding to the turning rollers.

Then, germ and bran layers are removed from the brown rice, leaving starching endosperm beneath. In a modern rice mill, a whitener is used to remove the germ and bran layers by a combination of cutting and tearing actions. The rice that has the husk and bran layers removed is called milled rice or white rice.

After the rice has been milled, broken kernels and other contaminants are removed from the whole milled grains (head rice). Controlled amounts of broken grains and nutrients are then mixed with the head rice as required by customers. Finally, the finish rice product is weighed and placed into packages for transport (Champagne, 2004).

1.4. Rice bran and oil

1.4.1. Rice bran composition

Rice bran from modern rice mills is a fine, powdery material composed of pericarp, testa (seed coat), aleurone, germ and some endosperm (Saunders, 1986). Typically, rice bran contains 15 - 20 % lipids, 12 - 16 % protein, 7 - 11 % crude fibre, 34 - 52 % carbohydrate and 7 - 10 % ash (McCaskill and Zhang, 1999). Total lipid content in rice bran depends on variety of rice and degree of milling (Hargrove, 1994). Since rice bran is rich in lipids, it is thus a commercially feasible feedstock for oil extraction.

In intact grain, rice lipids are in the aleurone and sub-aleurone layer and germ, while lipases are dormant and localized in the testa layer (Luh *et al.*, 1991). Once the bran is separated from kernel during milling, lipid and lipase come in contact, which promotes relatively rapid hydrolysis of lipids into free fatty acids, this results in a significant loss of triacylglycerols (TAGs) during refining of rice bran oil. Limited availability of stabilized rice bran and high capital costs for rice bran oil extraction have restricted the production of rice bran oil commerciality.

Lipids in cereals are generally distinguished into starch lipids and non-starch lipids (Morrison, 1988). The starch lipids are lipids inside or on surface of starch granules in endosperm and are composed mainly of monoacyl lipids. Although the starch lipids represent a relatively small proportion of the total lipids, they are involved in starch biosynthesis, starch degradation and are likely to affect starch functionality (Morrison, 1988). The non-starch lipids are the major lipids present in rice and include of membrane and spherosome lipids (Champagne, 2004). There are two important biological functions of lipids in cereals. First, lipids are used as energy reserves, mainly in the form of triacylglycerols and to a lesser extent diacylglycerols. Despite the fact that starchy endosperm is the main energy reserve of cereal grains, triacylglycerols are also important for sustaining the embryo before the arrival of sugar from

the starchy endosperm during early germination. Secondly, lipids in the form of polar lipids are structural components of cellular membranes, which alter extensively during germination (Clarke *et al.*, 1983).

Lipids in rice are predominantly found in organelles called spherosomes, also known more widely as oil bodies. Rice bran oil bodies have an electron dense boundary as revealed by electron micrographs. They are largest (≤ 1.5 µm) in the aleurone layer, followed by the subaleurone layer (≤ 1.0 µm) and smallest (≤ 0.7 µm) in the embryo (Juliano, 1983).

The major composition of crude rice bran oil is neutral lipids (Table 1.1). In comparison with other vegetable oils, crude rice bran oil tends to contain higher levels of non-triglyceride components, most of which are removed during refining processes. Free fatty acids, monoglycerides and diglycerides in rice bran oil are associated with enzymatic hydrolysis. The major phospholipids in rice are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (Fujino, 1978). The relative amount of unsaponifiable matter in rice bran oil is more than 4 %. Oryzanol, a mixture of ferulic acid esterified with phytosterols or triterpene alcohols comprises 2 % of the crude rice bran oil. Tocopherols and

tocotrienols represent about 0.04 % and 0.07 % of crude rice bran oil respectively.

Table 1.1 Composition of crude rice bran oil (Sayre and Saunders, 1990)

Component	% of total oil
Saponifiable lipids	**95**
Neutral lipids	85
Triglycerides	71
Diglycerides	3
Monoglycerides	5
Free fatty acids	2
Waxes	3
Glycolipids	6
Phospholipids	4
Unsaponifiable lipids	**4.2**
Phytosterols	1.8
Campesterol	0.51
Stigmasterol	0.27
β-Sitosterol	0.88
4-Methyl sterols	0.40
Triterpene alcohols	1.2
24-Methylene cycloartanol	0.49
Cycloartenol	0.48
Cycloartanol	0.11
Less polar compounds	0.80
Squalene	0.12
Tocopherols	0.04
Tocotrienols	0.07

Table 1.2 compares fatty acid composition of rice bran oil to that of peanut, soybean, and cottonseed oils. It can be seen that oleic and linoleic acids represent more than 80 % of the total fatty acid composition. The fatty acid composition of rice bran oil is similar to that of peanut oil, but slightly higher in saturation level than that of

soybean oil. The low linolenic acid content of rice bran oil makes it more stable to oxidation than soybean oil.

Table 1.2 Fatty acid composition of selected vegetable oils (McCaskill and Zhang, 1999)

Fatty Acid	Percentage			
	Rice Bran	Peanut	Soybean	Cottonseed
Myristic (14:0)	0.20	-	0.20	0.80
Palmitic (16:0)	15	8.1	11	27
Stearic (18:0)	1.9	1.5	3.9	2.0
Oleic (18:1)	42	50	23	18
Linoleic (18:2)	39	35	51	50
Linolenic (18:3)	1.1	-	6.8	-
Arachidic (20:0)	0.50	1.1	0.20	0.30
Behenic (22:0)	0.20	2.1	0.10	-

1.4.2. Rice bran oil processing

Processing of rice bran oil has some special features compared with other vegetable oils. The rice bran oil processing can be divided into two main parts; extraction of crude rice bran oil and refining process as shown in Figure 1.3. Rice bran is first stabilized to inactivate any lipolytic enzymes and to facilitate pellet or collet formation. The formation pellet increases solvent penetration rate leading to shorter oil extraction time. Rice bran oil is commercially extracted with hexane because of its efficiency and availability (Kao and Luh, 1991). Other solvents such as acetone, ethyl acetate, isopropanol and propane are also use for rice bran oil extraction

(Champagne, 2004). The process of refining food grade rice bran oil involves dewaxing, degumming, refining (neutralization), bleaching, winterization and deodorization (Nicolosi *et al.*, 1994). Neutralization is aimed to remove free fatty acids presented in the oil. A small excess of caustic soda solution (18 % NaOH, 4.5 M) is used to convert free fatty acids into hydratable sodium soaps, which are removable by centrifugation. The soap stock can be further treated with acid to yield high fatty acid oil for animal feed. High neutral lipid and unsaponifiable fraction loss to the soapstock also occurs, especially with rice bran oil containing high free fatty acids. Therefore, the level of free fatty acids in rice bran should be kept as low as possible to improve the efficiency of the refining as well as the quality of the refined oil.

During rice bran oil processing, oryzanol and tocotrienols in the unsaponifiable fraction of the crude rice bran oil have been reported to be lost by up to 90 % of the total amount in the crude oil (Nicolosi *et al.*, 1994). Obviously, this indicates the requirement to develop new rice bran processing methods that will increase both the oil recovery and the active phytochemical constituents. Rice bran oil is normally used in food, feed, specialty products and industrial applications such as salad dressing, mayonnaise, margarines, shortening, feed formulation, soaps, cosmetic products and pharmaceutical products.

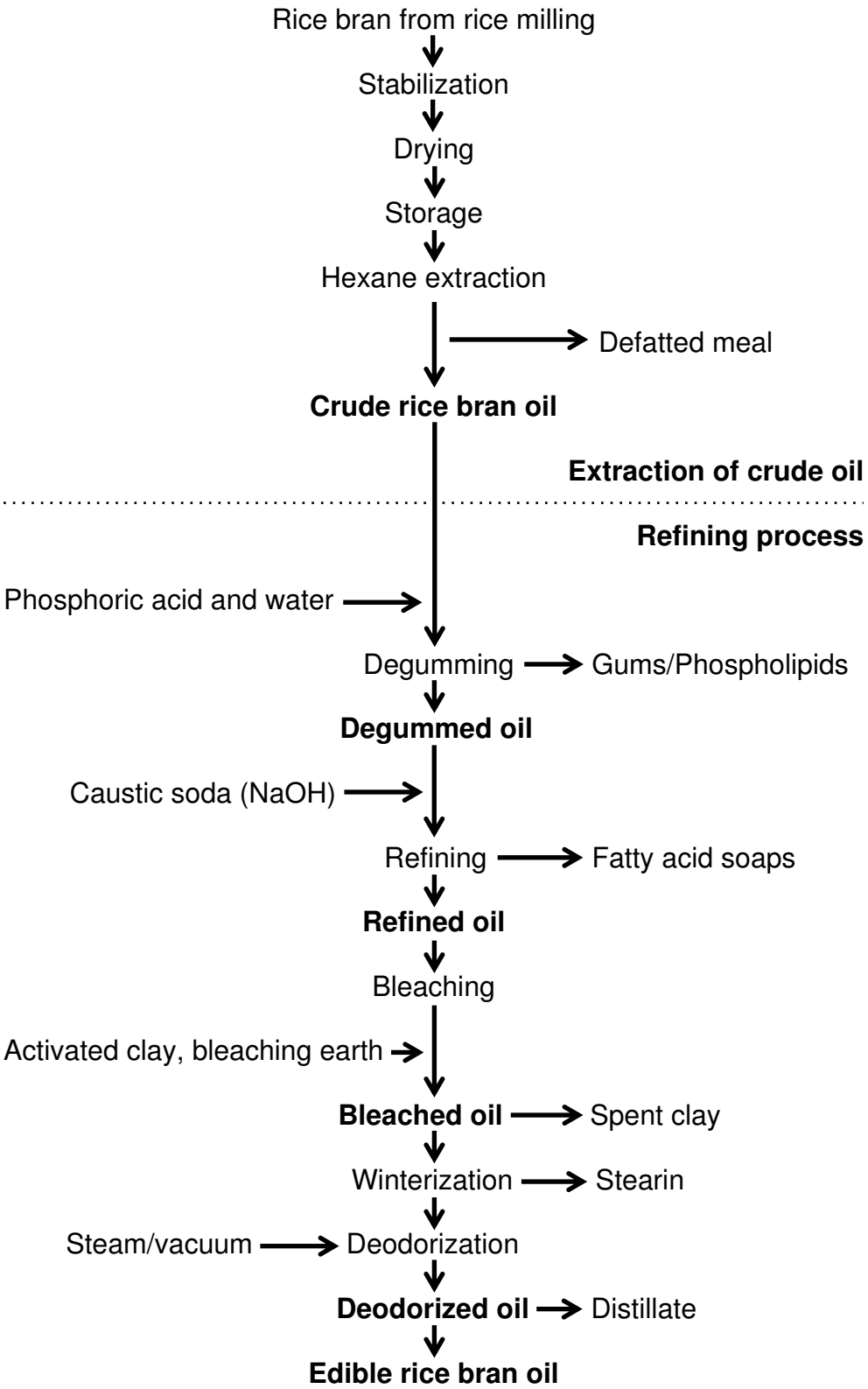


Figure 1.3 Rice bran oil production: crude oil extraction and refining (adapted from Nicolosi *et al.* (1994))

1.4.3. Rice bran stabilization

Rice bran is abundant in various active enzymes that contribute to rice bran instability and limit rice bran industrial usability. These include lipase, lipoxygenase and peroxidase. In addition to endogenous enzymes, microbial enzymes also causes deterioration of rice bran (Luh *et al.*, 1991). Once the bran is removed from the kernel, lipid substrate and enzymes are brought together and enzymatic hydrolysis proceeds rapidly. Microorganisms on the surface of the kernel would then gain access to rice bran oil.

Typically, rice bran oil contains 1.5 - 2.0 % free fatty acids (FFAs), at the time of milling. FFAs have been reported to increase 5 - 7 % in a single day and up to 70% in a month. It is recommended that crude rice bran oil should contain FFAs less than 5 % for economic recovery of refined edible oil (Saunders, 1986). Therefore, rice bran stabilization (inactivation of the active enzymes) should be done immediately after milling. A number of rice bran stabilization methods have been published previously (Nasirullah *et al.*, 1989; Prabhakar and Venkatesh, 1986; Sayre *et al.*, 1982). These methods include use of chemicals, cold storage and heat treatment. The heat treatment appears to be the most commercial potential method. Three types of heat treatment procedures have been used including: dry heating, added moisture heating and retained moisture heating (Sayre *et al.*, 1982).

A simple and inexpensive method of dry heat treatment was performed by open-pan roasting rice bran in a hot air oven at 110 ºC for 10 minutes (Nasirullah *et al.*, 1989). This primarily inactivates lipase by removing water required for hydrolysis. FFA content of the oil extracted from the heat-treated bran increases slightly (from 4.8 % to 5.0 %) during 7 day of storage. However, heating rice bran for 20 minutes at 110 ºC was suggested for proper inactivation of lipase activities (Luh *et al.*, 1991). Disadvantages of this method are reduction of nutritional components in rice bran, high energy consumption required for considerable amount of moisture removal, and incomplete or reversible inactivation of enzymes (Luh *et al.*, 1991).

Heating in the presence of moisture at high temperature is much more effective at inactivating heat-stable enzymes in rice bran such as peroxidase than the dry heating method (Luh *et al.*, 1991). Nevertheless, requirement and cost of a steam generating system severely restrict the added moisture stabilization (Sayre *et al.*, 1982).

The retained moisture heat treatment using an extrusion cooking has been suggested to be an effective method that produces stable rice bran (Randall *et al.*, 1985). This method uniformly exposed the bran to high temperature for a short time. The optimum process was developed by extruding 500 kg/hr of 12 - 13 % moisture

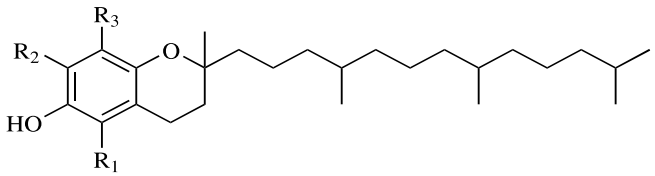
bran at 130 ºC and holding for 3 minutes at 97 - 99 ºC before cooling. The stabilized rice bran contained 6 - 7 % moisture and was in the form of small flakes. The bran showed no significant increase in FFA content for at least 30 - 60 days. Since the retained moisture heating uses only electricity and does not require an external steam supply, it is simple to install and operate.

1.4.4. Rice bran phytochemicals

Among many phytochemicals associated with the unsaponifiable fraction of rice bran oil, tocochromanols (tocotrienols and tocopherols) and oryzanol are of interest because of their natural antioxidant properties. These components make rice bran oil specialty oil for niche markets. In recent years, research has been conducted to study the health benefits of these phytochemicals as co-products of rice bran oil processing (Chen and Bergman, 2005; Rogers *et al.*, 1993).

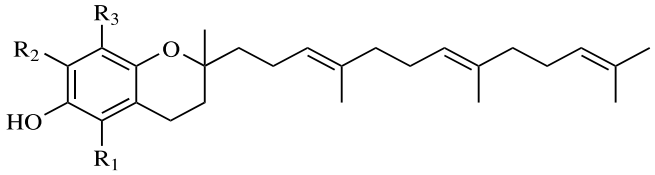
Rice bran oil contains tocotrienols and tocopherols (Figure 1.4), members of the vitamin E family that possess a common hydroxychromane ring and a terpenoid side chain. The four common isomers of tocotrienols and tocopherols in nature have been found as α, β, γ and δ (Shin and Godber, 1994). It has been claimed that α - tocotrienol has a more potent antioxidant activity than α - tocopherol (Suzuki *et al.*, 1993). Tocotrienols were reported to reduce serum

triglycerides and cholesterol levels (Qureshi *et al.*, 2001), and suppress carcinogenesis (Wada *et al.*, 2005). In addition to the antioxidant properties of tocochromanols, other biological functions are proposed, such as maintaining stability of cellular membrane, helping in signal transduction and being involved in cell proliferation and differentiation (Suzuki *et al.*, 1993).



Tocopherols (T)

	R_1	R_2	R_3
α-T(3)	CH_3	CH_3	CH_3
β-T(3)	CH_3	H	CH_3
γ-T(3)	H	CH_3	CH_3
δ-T(3)	H	H	CH_3



Tocotrienols (T3)

Figure 1.4 Structure of tocopherols and tocotrienols (Champagne, 2004)

Oryzanol was first discovered as a single compound (melting point of 138.5 ºC) from rice bran oil in 1954 by Kaneko and Tsuchiya (1954), who called it oryzanol. Later, it was found to be a group of phytosterols or triterpene alcohols esterified to ferulic acid (4-hydroxy-3-methoxy cinnamic acid). These compounds were separated by RP-HPLC and identified by mass spectrometry as cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campersteryl ferulate, β-sitosteryl ferulate, and cycloartanyl ferulate

(Rogers *et al*., 1993). From the study of Xu and Godber (1999), 10 components of purified oryzanol were identified by RP-HPLC with the aid of GC/MS. The major components of oryzanol were reported as cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and campesteryl ferulate (Figure 1.5).

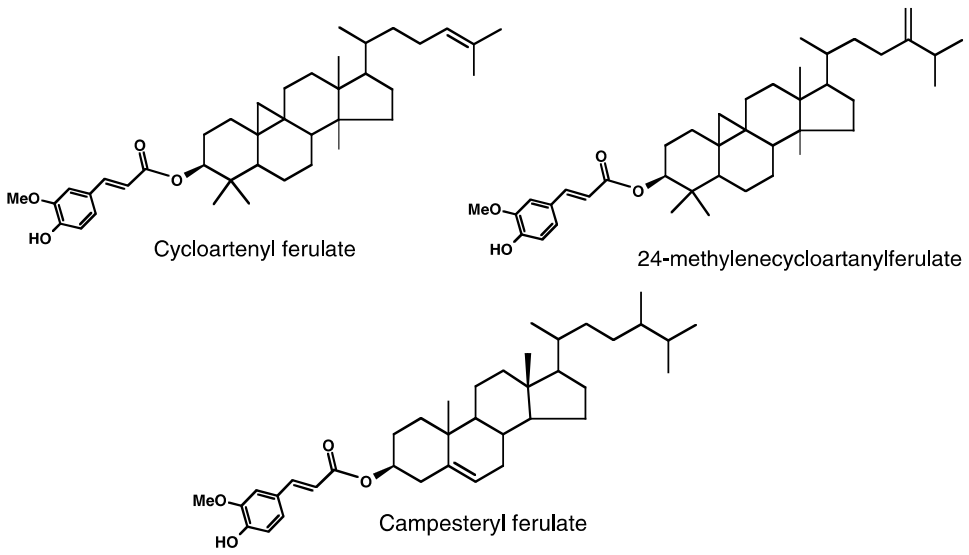


Figure 1.5 Structure of three major oryzanol components (Xu and Godber, 1999)

During refining process, oryzanol can be first isolated and purified from soap stock as a valuable by-product. Then it may be either added back into the refined rice bran oil or used as an additive in other functional foods. Crude rice bran oil contains about 2 % or more oryzanol depending on the refining method. For degummed oil, oryzanol is reduced to 1.7 %. Physical refined oil and alkaline refined oil contains 1.0 - 1.5 % and 0.1 % oryzanol respectively (Orthoefer, 1996).

The structures of major oryzanol components are similar to that of cholesterol suggesting that this similarity may be contributed to the cholesterol lowering effects of oryzanol. Oryzanol has been reported to reduce plasma cholesterol, low density lipoprotein (LDL) cholesterol, cholesterol absorption and early atherosclerosis (fatty streak formation) (Ausman *et al.*, 2005; Lichtenstein *et al.*, 1994). A biological function of oryzanol is unclear from the current literature; however, it may play roles in maintaining function of plant cell membranes and regulating membrane fluidity.

Levels of tocochromanols and oryzanol in different rice fractions and commercial available rice bran oil are listed in Table 1.3. Levels of these phytochemicals in brown rice and crude rice bran oil can vary widely. Heinemann *et al.* (2008) reported total tocochromanols and oryzanol of 16 - 24 mg/kg and 190 - 246 mg/kg respectively in different varieties of brown rice. They also found that γ-tocotrienol was the most abundant isomer in *indica* rice, while α-tocotrienol was the highest level in *japonica* rice.

In addition, commercial crude rice bran oil contained 550 mg/kg tocochromanols and 21,100 mg/kg oryzanol, while physical refined rice bran oil contained 180 mg/kg and 2,300 mg/kg respectively (Hoed *et al.*, 2010).

Table 1.3 Tocochromanol and oryzanol concentrations (mg/kg) in raw rice bran, brown rice and crude rice bran oil (crude RBO) and commercially available refined rice bran oil (refined RBO)

Phytochemical [1]	Rice bran [2]	Brown rice [3]	Crude RBO [4]	Refined RBO [5]
δ -T3	7	1	36	ND - 104
β/γ-T3	120	12	599	62 - 975
α-T3	38	4	191	ND - 86
δ-T	2	0	10	ND - 40
β/γ-T	41	4	206	16 - 358
α-T	63	6	316	ND - 218
Total T3	165	17	826	72 - 1,157
Total T	106	11	532	16 - 452
Total	272	27	1,358	88 - 1,609
Oryzanol	3,102	310	15,508	115 - 787

[1] T3 = tocotrienol; T = tocopherol; Total = total tocochromanols (T3+T)
[2] Adapted from Shin and Godber (1996)
[3] Calculated based on 10 % rice bran yield as determined by Shin and Godber (1996)
[4] Calculated based on 20 % lipid in rice bran as determined by Shin and Godber (1996)
[5] Data from Rogers *et al.* (1993); content of commercial refined rice bran from 5 different manufacturers. ND = Not detected

Concentration and types of rice bran tocochromanols depend on various factors including method of extraction (Hu *et al.*, 1996), rice bran stabilization and subsequence storage (Shin *et al.*, 1997), degree of milling (Lloyd *et al.*, 2000) and rice growing environment (Bergman and Xu, 2003). Increasing solvent to bran ratio and extraction temperature was reported to increase amount of crude oil, tocochromanols and oryzanol (Hu *et al.*, 1996). Isopropanol (3:1, solvent/bran ratio at 60 ºC) extracted significantly more (P<0.05) tocochromanols (171 mg/kg rice bran), but similar (P>0.05) amounts of oryzanol (2,930 mg/kg rice bran) relative to hexane

(tocochromanols 157 mg/kg rice bran and oryzanol 2,847 mg/kg rice bran). Extruded rice bran at 110 ºC and 140 ºC contained 304 mg/kg and 274 mg/kg tocochromanols, and 3,132 mg/kg and 2,981 mg/kg oryzanol respectively (Shin *et al.*, 1997). They also concluded that γ-tocotrienol was more stable than other tocochromanol isomers during a year storage period. In addition, it was reported that the concentration of oryzanol in rice bran were about 10 times higher than that of tocochromanols (Heinemann *et al.*, 2008).

1.5. Oil bodies

Multicellular organisms store food reserves in special organs before a dormant period or living in adverse environmental conditions. The food reserves can be stored in forms of fats/oils, carbohydrate, and protein. Among these, fats/oils are the most efficient energy source as they are more compact and provide energy almost twice of those stored in starch per unit weight (Buchanan *et al.*, 2000). Since triacylglycerols are relatively inert, they can be stored in large quantity without risking interaction with other cellular components. Triacylglycerols are separated as lipid droplets because of their hydrophobicity and insolubility in water. Plant seeds store cellular fats and oils in the form of triacylglycerols (TAGs) in distinct spherical organelles called oil bodies (Huang, 1996; Murphy, 2001a). Plant oil bodies serves as an energy source and membrane lipid building blocks for embryo germination and post-germinative growth.

1.5.1. Oil body composition

Oil bodies are composed of a neutral lipid core surrounded by a monolayer of phospholipids (PLs) and partially embedded protein (Figure 1.6). The major protein surrounding the oil body is called oleosin (Tzen *et al.*, 1993). Average size of oil bodies are in range of 0.2 - 2.5 µm in diameter (Huang, 1992). The sizes of oil bodies can differ and vary due to nutrient supply and environmental conditions of the parent plants; they also seem to vary depending on their intra-grain locations (aleurone layer, embryo or endosperm).

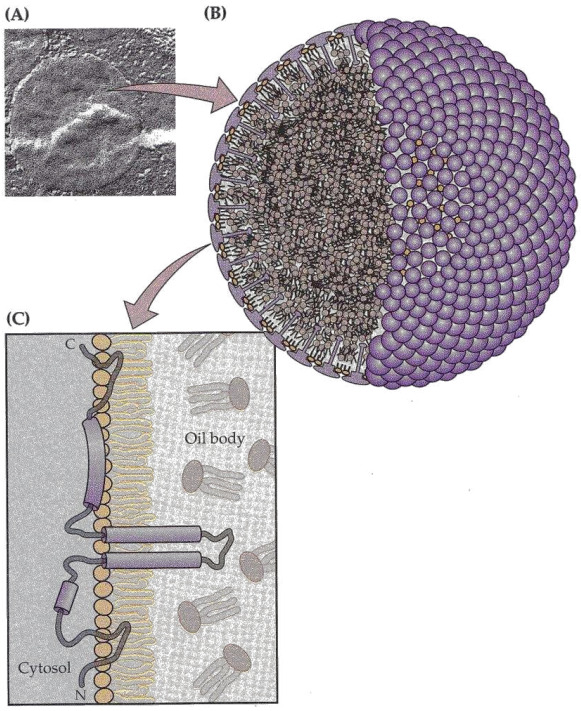


Figure 1.6 Structure of oil body (A) transmission electron micrograph of an oil body, (B) Model of a maize oil body with oleosin molecules (11 nm long hydrophobic stalk) attached to an amphipathic and hydrophilic globular structure on the outer oil body surface and (C) Proposed model of the conformation of a maize 18 kDa oleosin with cylinders represented as helices (Buchanan *et al.*, 2000)

In general, the oil body composition from plant seeds (rape, mustard, cotton, flax, maize, peanut and sesame) consists of neutral lipid (94 - 98% w/w), phospholipids (0.5 - 2%), protein (0.5 - 3.5%) and free fatty acids (0.1 - 0.4%) (Tzen *et al.*, 1993). PLs in oil bodies from various species have been reported to contain phosphatidylcholine, PC (41.2 - 64.1 %) as the major components, followed by phosphatidylserine, PS (18.3 - 33.1 %), phosphatidyl-inositol, PI (6.9 - 20.9%) and phosphatidylethanolamine, PE (2.8 - 15.8%) respectively (Tzen *et al.*, 1993).

Oleosins are proteins with low-molecular mass (15 - 26 kDa), depending on isoforms and plant species (Qu *et al.*, 1986). Oleosin and associated proteins may play important roles in biological functions related to oil body synthesis and degradation. Additionally, oleosins may act as receptors on the surface of oil bodies for binding of newly synthesized lipase during germination (Huang, 1996). Oleosins contain three basic structural domains including 1) an amphipathic N-terminal domain (40 - 60 amino acids); 2) a central hydrophobic domain (68 - 74 amino acids with a “proline knot” consisting of three prolines and one serine residue); and 3) an amphipathic C- terminal domain (33 - 40 amino acids).

Transmission electron micrographs have shown that the highest concentrations of oil bodies are located within the aleurone,

sub-aleurone and germ, and not the starchy endosperm of oat and rice grain (Chuang *et al.*, 1996; White *et al.*, 2006). Rice oil body diameters were observed between 0.5 - 1 µm (Wu *et al.*, 1998). Two oleosin isoforms of molecular masses 16 and 18 kDa were found on the surface of oil bodies of embryo and aleurone layer of matured rice (Wu *et al.*, 1998) and rice embryos (Chuang *et al.*, 1996).

Oil bodies *in vivo* and *ex vivo* are remarkably stable and do not aggregate or coalesce (Huang, 1992). The oil bodies maintained their structure as discrete organelles with hydrophilic surface. Both oleosins and PLs are required to stabilize the oil bodies (Tzen and Huang, 1992). Physical stability of oil bodies has been reported to depend on two factors:

(1) **Strengthened surface layer** provided by phospholipids and proteins at the surface of oil bodies. Surface strength rather than simply steric hindrance prevents closely associated or aggregated oil bodies from coalescence *in vitro*. *In vivo*, surface strength allows oil bodies to be highly compressed against each other without coalescence and remain as individual entities against physical forces during seed desiccation (White *et al.*, 2008). Removal of oleosins by trypsin digestion induced aggregation and coalescence of adjacent oil bodies. However, treatment of oil bodies with phospholipases has

no effect on the stability of the oil bodies, apparently due to oleosins that shield PLs from being hydrolyzed (Tzen and Huang, 1992).

(2) **Electrostatic repulsion** provided by the negatively charged surface of the organelle. The oleosins, PLs and small amount of FFAs present on the oil body surface interact among themselves and with the TAG core. Oleosin orient the molecule in the way that negative charges are exposed to cytosol, whereas the positive charges face the interior to the TAG matrix of the oil bodies. These positively charged residues also interact with the negatively charged PLs (phosphatidylserine and phosphatidylinositol) and FFAs. These make overall charges of the oil body surface negative at pH 7.0 and prevent oil bodies from aggregating and coalescing (Huang, 1992).

1.5.2. Oil body biogenesis

A number of oil body formation models have been proposed (Buchanan *et al.*, 2000; Huang, 1996; Murphy and Vance, 1999). Oil bodies in seed have been suggested to bud out from endoplasmic reticulum (ER) membrane where high activities of TAG biosynthesis enzymes are found (Murphy, 2001b). Maize oil bodies are produced by budding of TAG accumulated between PL bilayer of the ER (Figure 1.7). Simultaneously, oleosins are synthesized on ribosomes attached to the ER. The oleosins would either localize or insert

directly into the budding TAG particle. Once the TAG matrix is surrounded by a monolayer of PL and oleosins, they are released into cytosol as a matured oil body (Huang, 1996).

1.5.3. Oil body degradation

During seed germination and postgerminative seedlings, TAGs were mobilized to provide carbon source and chemical energy within a specialized peroxisome called glyoxysome. Because of the hydrophobicity of TAGs stored in the oil bodies, they must be hydrolyzed to fatty acids before they can be used for metabolism. Lipase is synthesized on free polyribosomes and then binds specifically to the oil bodies (Figure 1.7). Hydrolysis of TAGs produces fatty acids that are further broken down via β-oxidation. Hydrolyzed products in the form of acyl-CoA may be converted via glyoxylate cycle and gluconeogenesis to carbohydrate (Buchanan *et al.*, 2000).

From the study of scutellum of maize postgerminative seedling, lipase activities increased simultaneously with the decrease in stored TAGs. After 6 days of imbibition, most of the TAGs had been degraded. At the late stage of lipid mobilization, fusion of oil bodies and an expanding vacuole could be observed under electron microscopy (Wang and Huangs, 1987). During or after the oil body degradation, lipase may follow the degraded oil bodies or remain in

cytosol. Oleosins degrade rapidly with TAG depletion. The PLs and lipase may fuse with the enlarging vacuole membrane and are degraded afterwards (Huang, 1992).

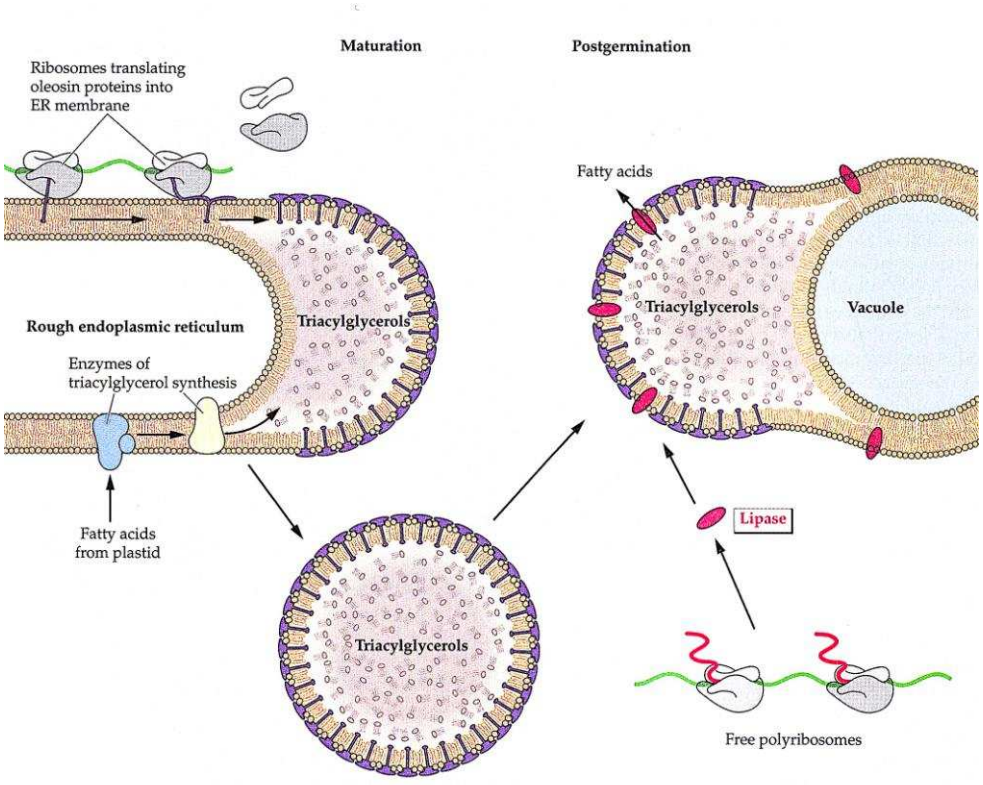


Figure 1.7 Synthesis and degradation model of an oil body in a maize embryo during seed maturation and postgermination proposed by Buchanan *et al.* (2000)

The model of synthesis and degradation of oil bodies in other seed species may be different from the maize oil bodies. In germinating sesame seedling, the germination process may last for several days. All of the oil bodies were not mobilized simultaneously. Remnant oil bodies, i.e. those not yet mobilized for breakdown, are not altered in integrity (ratio of TAG and protein content remains constant) and preserved intact so that the energy from the stored

lipids can be supplied throughout the whole process (Tzen *et al.*, 1997). In postgerminative seedlings of rice embryos, 60 % of the stored TAGs were not used. It seems that the oil bodies in the germ of the grain are mobilized, but those in the aleurone and sub-aleurone layers remain intact, preserving their TAG molecules. However, a function of the remaining intact oil bodies in rice aleurone layers was not clarified. They may perform other biological function(s) other than energy storage (Wu *et al.*, 1998).

1.6. Emulsions

An emulsion is described as a dispersion of droplets of one liquid in another liquid, which is incompletely miscible such as oil and water (McClements, 1999). A system consists of oil droplets dispersed in water phase is called oil-in-water (O/W) emulsion or vice versa (W/O emulsion). Since emulsions are typically thermodynamically unstable systems, they tend to separate into oil layer (lower density) and water layer (higher density). Therefore, amphiphilic molecules of emulsifiers are added to the emulsions to decrease interfacial tension between the oil and water phase and form a protective membrane that prevents droplets from aggregating and coalescing with neighboring droplets (Akoh and Min, 2008). Instability of emulsion occurs through various physical mechanisms including creaming, sedimentation, flocculation and coalescence.

1.6.1. Characterization of emulsion properties

Some of important properties of emulsions are determined by dispersed phase volume distribution (proximate analysis and density), droplet size distribution (light scattering and microscopy), microstructure (light and electron microscopy), droplet-droplet interaction (creaming and sedimentation, droplet charges/zeta-potential and droplet crystallization), emulsion rheology (viscometer and rheometers) and interfacial properties (tensiometers). These characteristics influence appearance, texture, taste, shelf-life and sensory properties of the end products (Akoh and Min, 2008).

1.6.2. Oil body suspension

Oil body recovery is an alternative approach to recover oil that is based on non-toxic, non-volatile solvent. This aqueous-based oil body recovery method extracts oil and lipophilic phytonutrients without severely degrading and changing their functionalities from soybean, sunflower and oat (Fisk *et al.*, 2006; Fisk and Gray, 2011; White *et al.*, 2006; White *et al.*, 2009). Natural properties of oil body structure made it easier to emulsify and more stable than the bulk rice bran oil. Production cost required for emulsifiers and homogenization would be reduced. Additionally, the presence of natural antioxidants in the oil bodies could improve stability during processing, storage, transport and utilization of the final lipid-based products.

Properties of oil body suspension are normally analyzed using zeta-potential, particle size, and creaming or turbidity. Oil bodies are negative charges near physiological pH (pH 7) (Chuang *et al.*, 1996; Tzen *et al.*, 1993). The negative charges surface provides electrostatic repulsion that maintains the stability of oil body suspension. At pH values well away from isoelectric point (pI or the pH where species have a net charge of zero), oil bodies in suspension were relatively small and stable to creaming. However, at pH values close to the isoelectric point or in the presence of salt, oil bodies have relatively poor stability and tend to aggregate.

In addition, soybean oil bodies have been reported to have similar or more improved physical and thermal stability compared to emulsified soybean oil (Iwanaga *et al.*, 2007). The oil body suspension was stable to aggregation and creaming at low ionic strength (NaCl ≤ 25 mM) and heat (30 - 60 ºC). Oil body suspensions appear to be less efficient substrates for lipolysis than emulsified oil due to the presence of oleosins and phospholipids on the oil body surface, and, at least in the study cited, the smaller surface/volume ratio offered by oil bodies to lipases (Beisson *et al.*, 2001).

1.7. Lipid hydrolysis and oxidation

Lipid deterioration can be divided into non-enzymatic reactions (e.g. autoxidation) and enzymatic reactions (Lehtinen and Laakso, 2004). The non-enzymatic reactions are typically slow at ambient temperature and pH values during cereal processing and are related to oxidation and isomerization of carbon-carbon double bond structure in the lipids. In contrast, the enzymatic reactions have been extensively studied and reported in the literature, since they importantly influence stability and storage of cereal and food products. The enzymatic reactions are mostly reported to be associated with hydrolytic or oxidative pathways.

Once the bran is removed from kernel during milling, lipid hydrolysis and oxidation occur rapidly. Hydroperoxides produced from the oxidation further decompose to secondary oxidation products as shown in Figure 1.8. Crude rice bran oil that is not passed through a refining process is therefore, highly unstable due to high levels of very active lipases.

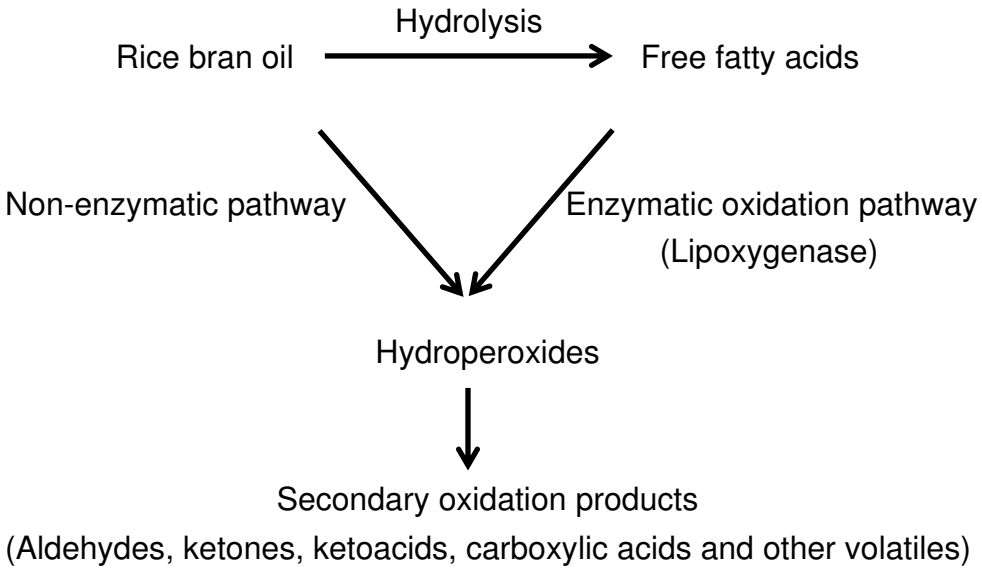


Figure 1.8 Pathways of hydrolytic and oxidative deterioration of rice bran oil (Champagne, 1994)

1.7.1. Non-enzymatic reactions

Autoxidation is typically initiated by factors such as radicals, high energy electron magnetic radiation, or transition metal-ions. Photooxygenation is triggered by photons from visible light, which in turn convert ground state molecular oxygen into singlet oxygen. Autoxidation is a free-radically driven chain reaction, characterized by three steps: initiation, propagation and termination (Frankel, 2005). Once the reaction is initiated, radicals formed from the reaction will enhance and continue the reaction further.

Autoxidation is inhibited or retarded by antioxidants. Chain breaking antioxidants interfere with chain propagation and initiation

and induce decomposition of hydroperoxides by reducing themselves to relatively stable or inactive products. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), prophyl gallate (PG) and *tert*-butylhydroquinone (TBHQ) are used to inhibit the autoxidation and rancidity. However, natural antioxidants e.g. vitamin E (tocopherols and tocotrienols), oryzanol and other phenolic compounds presented in food are receiving considerable attention. They not only provide antioxidant activities to food, but also have additional effects such as health promotion.

Metal inactivators are other classes of antioxidants that chelate metal ions, which promote the initiation and decomposition of hydroperoxides. They function by suppressing redox reaction or preventing formation and decomposition of hydroperoxides. Common inactivating chelating compounds include ethylenediamine tetraacetic acid (EDTA), citric acid, phosphoric acid, polyphosphates and phytate. In addition, pigments such as carotenoids are compounds that can absorb light energy without formation of radicals and can deactivate photooxygenation by quenching reactive molecules into non-reactive form.

Isomerization of unsaturated fatty acids does not occur at ambient processing conditions. However, high temperature during deodorization (> 245 ºC) and heating at frying temperature (180 ºC

for 8 hours) with presence of oxygen leads to an increase of trans-linolenic acid (tr-18:2) in rice bran oil (Mezouari and Eichner, 2008).

1.7.2. Enzymatic hydrolysis

Hydrolysis of acylglycerols to fatty acids and glycerols is catalyzed by lipase enzymes. Lipase extracted from rice bran (40 kDa) has been reported to have an optimum pH for catalysis of between 7.5 - 8.0, and the optimum temperature at about 37 ºC. The enzymes are activated by the presence of a low level of calcium ions (less than 0.01 M) and inhibited by EDTA. Rice bran lipase preferentially hydrolyzes fatty acid ester bonds in TAG at the 1,3-position (Aizono *et al.*, 1973). Phospholipases are enzymes that catalyze the hydrolysis of phospholipids. Multiple forms of phospholipases in plants exist i.e. phospholipase A1, A2, C and D (Wang, 2001).

1.7.3. Enzymatic oxidation

Lipoxygenases (linoleate oxygen oxidoreductase or LOX) catalyzes oxidation of free polyunsaturated fatty acids containing cis,cis-1,4-pentadiene moieties, such as linoleic acid and linolenic acid, into conjugated hydroperoxide fatty acids (Gardner, 1991). Although lipoxygenase catalyze lipid oxidation after lipid hydrolysis, this oxidative process plays important biological roles in plants and animals. Lipoxygenase pathway in plants is activated by wounding and pathogen attack (Gardner, 1991). The lipoxygenase pathway

also involves in the biosynthesis of jasmonic acid and other metabolites relating in many biological activities such as protein induction, protein degradation, secondary metabolite induction, senescence, and growth inhibition (Gardner, 1991).

Three multiple isoforms of lipoxygenase (LOX-1, LOX-2 and LOX-3) was found in embryo and bran of rice grain, LOX-3 being the major form (Ida *et al*., 1983). Lipoxygenase activities in rice was inactivated by heating for 10 minutes at 90 ºC (Ratchatachaiyos and Theerakulkait, 2009). Oxidation of linoleic acid by rice lipoxygenase produced 9- and 13-hydroperoxide linoleic acids. (Ida *et al*., 1983; Suzuki *et al*., 1996).

Products from the oxidation LOX are further metabolized by various plant enzymes such as hydroperoxide dehydrase, hydroperoxide lyase, hydroperoxide isomerase, hydroperoxide-dependent peroxygenase, and hydroperoxide-dependent epoxygenase (Gardner, 1991). Decomposition products of lipid hydroperoxides include carbonyl compounds, alcohols and hydrocarbon of various chain lengths (Frankel, 2005). These compounds are involved in quality and stability of food products. In oat, peroxygenase and epoxide hydrolase were reported to break down hydroperoxides to hydroxy fatty acids, which may contribute to bitter taste (Hamberg and Hamberg, 1996).

It is known that lipid oxidation significantly impacts nutritional values, sensory, rheological properties, texture and stability of food (Frankel, 2005; Hu *et al.*, 2003; Jacosen, 1999). “Rancid” is a term commonly used to describe off odours and off-flavours caused by lipid oxidation. Sensory evaluation is not often proportional to chemical analysis values and may vary in different foods (Jacosen, 1999). Volatile compounds, the secondary products from lipid oxidation, have been used widely to study the extent of lipid oxidation. Several procedures have been proposed to identify and quantify volatiles such as simultaneous distillation/extraction (Parrado *et al.*), Fourier transform infrared spectroscopy (FTIR) and headspace - solid phase microextraction (SPME) (Beltrán *et al.*, 2011; Widjaja *et al.*, 1996). Volatile compounds that show significant correlation ($r > 0.9$) with the oxidation are identified as nonanal, hexanal, 2-heptenal, octane, 2-pentylfuran, 2-propenal, c,t-2,4-heptadienal, t,t-2,4-heptadienal and heptanal (Frankel, 2005). Hexanal, derived from linoleic acid, is commonly used as a marker for lipid oxidation since it increases the most rapidly and is easily detected by headspace gas chromatography (Frankel *et al.*, 1989; Shin *et al.*, 1986).

1.8. Water activity

The water activity (a_w) of a system highly influences lipase activity, lipid oxidation rate and the stability of foods (Nelson and Labuza, 1992). Water activates enzymatic reactions and also affects

equilibrium of the reactions. Figure 1.9 shows that decreased water activity slows the growth of microorganisms, enzyme activity (especially involving hydrolase) and non-enzymatic browning. However, the rate of lipid oxidation increases at very low (a_w < 0.2) and high water activity (a_w > 0.4) compared with the rate at an intermediate values (a_w = 0.2 - 0.4) (Labuzaa and Dugan, 1971).

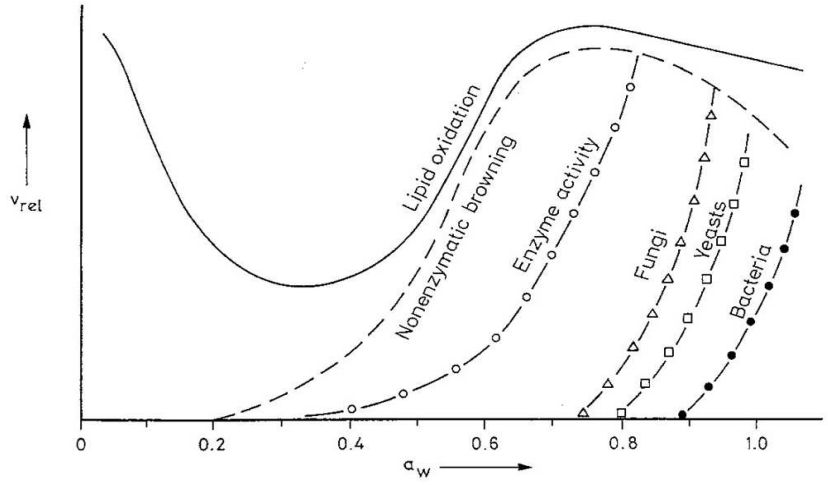


Figure 1.9 Food stability as a function of water activity (Labuzaa and Dugan, 1971)

V_{rel} = relative reaction rate; a_w = water activity

Therefore, control of initial moisture content or water activity is crucial in many food systems. In foods susceptible to lipid oxidation like rice, the initial moisture content is typically lowered to 12 - 13% before storage (Champagne, 2004). Milled rice with moisture content less than 14 % exposed to air at mid-range of relative humidity (40 - 65 %) has been reported to have minimal breakage during storage at 30 ºC than at low (< 40 %) and high (> 75 %) relative humidity levels (Siebenmorgen *et al.*, 1998).

1.9. Objectives

The purpose of this study is to process rice bran in an innovative way to yield added value products enriched in oil and phytonutrients. To our knowledge, this is the first attempt to recover and characterize oil bodies from rice bran. Also, a physical association between tocochromanols and oryzanol with rice bran oil bodies has never been established before. Stability of rice bran and its oil bodies are also studied during storage at different temperatures and relative humidity, and after heat treatment.

The objectives of this study are to:

1. Determine the location of oil bodies in rice grain
2. Source rice bran for this study
3. Establish the impact of heat treatment of rice bran on the morphology of oil bodies *in vivo*
4. Develop a method to recover and wash oil bodies from rice bran
5. Establish the physical properties of oil bodies *ex vivo*
6. Measure the carry-over of target phytochemicals in oil bodies recovered from rice bran
7. Investigate the impact of rice bran storage conditions on the quality of oil bodies *ex vivo*
8. Evaluate the viability of the oil bodies recovered from fresh or rancid rice bran

2. MATERIALS AND METHODS

2.1. Materials

Basmati brown rice (*Oryza sativa*) harvested during crop season 2007 from India was purchased from East End Food plc. (Birmingham, UK). The grains were milled using NW1000 Turbo rice milling (Natrawee technology, Bangkok Thailand). The fresh rice bran in this study referred to the freshly milled rice bran derived from the brown rice grains that had been stored for more than one year. The fresh bran was sieved through a 600 µm screen to remove broken grains, hull fragments, paddy kernels and other materials. All chemicals were analytical grades or higher, and obtained from Fisher UK (Loughborough, UK) unless otherwise stated.

2.2. Rice bran stabilization

2.2.1. Hot air drying

Thin layer of freshly milled rice bran (less than 0.5 mm in height) was heated on the pre-heated aluminum tray in a hot air oven at 110 ºC for 0, 1, 5, 10 and 20 min.

2.2.2. Extrusion stabilization

The extrusion was performed in a pilot-scale, modular twin-screw extruder (Prism TSE 24 MC, Thermo Electron Corporation,

UK). The screw diameter was 23.6 mm. The screw speed was 200 rpm and the pressure was held at 5 Bar with torque per shaft 10 Nm. The feed rate was maintained at 2 kg/h. The temperatures of zones 2 - 10 were ranged from 80 ℃ to 130 ºC. The extruded rice bran was held for 3 min before it was forced through a round die of 3 mm in diameter.

2.3. Oil body recovery and washing

2.3.1. Mortar and pestle oil body recovery

Rice bran (50 g) in 250 ml distilled water (conductivity 0.5 µS/cm) was mixed on a roller mixer at 50 rpm for 1 h before it was ground for 5 min. The slurry was filtered under vacuum through three layers of cheesecloth and centrifuged (10,000 x g for 20 min at 5 ºC). The creamed oil body layer floating on the top of the homogenate was carefully collected and labeled as mortar and pestle-crude oil bodies (MP-COB).

2.3.2. Water-based oil body recovery

Rice bran (50 g) in 250 ml distilled water (conductivity 0.5 µS/cm) was mixed on a roller mixer at 50 rpm for 1 h before homogenization in a blender (Krups, UK) for 2 min at the maximum speed. The slurry was filtered under vacuum through three layers of cheesecloth. The filtrate was then centrifuged at 10,000 g for 20 min at 5 ºC. The creamed oil body layer floating on the top of the

homogenate was carefully collected and called crude oil bodies (COB). For washing, crude oil bodies were resuspended in distilled water or 9 M urea (pH 9) at a ratio of 1:4, w/v (oil body : washing solution), vortexed vigorously and centrifuged at 10,000 g for 20 min at 5 ºC before the recovery of the washed oil body pad. The oil bodies were washed three more times with distilled water. The washed oil body pads with distilled water or 9 M urea were called water-washed oil bodies (WWOB) and urea-washed oil bodies (UWOB) respectively. The oil bodies were stored at 4 ºC for a maximum of 2 days prior to use.

2.3.3. Alkali-based oil body recovery

Rice bran (50 g) was homogenized in 250 ml 0.1 M Tris-HCl pH 8, containing 1 mM EDTA by using Silverson high shear laboratory mixer (Silverson Machine Ltd., Buckingham, UK) with Emulsor screen at 6,000 rpm for 40 sec. The slurry was filtered under vacuum through three layers of cheesecloth. The filtrate was then centrifuged at 10,000 g for 20 min at 5 ºC. The creamed oil body layer floating on the top of the homogenate was carefully collected and called alkali-crude oil bodies (alkali-COB). The crude oil bodies were further purified by washing with 250 ml of freshly prepared 0.1 M $NaHCO_3$, containing 1 mM EDTA with Silverson with Emulsor screen at 6,000 rpm for 5 sec. The slurry was filtered and centrifuged under the same conditions as those described above. The creamed

oil body layer was recovered, resuspended and centrifuge one more time in the same alkali solution. The recovered cream layer was resuspended and centrifuged twice in 250 ml of 1 mM Tris-HCl pH 8, containing 1 mM EDTA. The final cream layer were carefully collected and called alkali-washed oil bodies (alkali-WOB). The oil bodies were stored at 4 ºC for a maximum of 2 days prior to use.

2.3.4. Enzyme-assisted oil body recovery

Cell wall digesting enzymes including cellulase from *Trichoderma reseei* (≥ 1 units/mg solid), cellulase from *Aspergillus niger* (≥ 0.3 units/mg solid) and papain from *Carica papaya* (≥ 3 units/mg solid) were used in this study. An enzyme solution was made by mixing enzyme powder (1 g) with 20 ml 50 mM sodium citrate buffer (pH 4.8) in a dialysis membrane overnight in a large beaker containing 2 L 50mM sodium citrate buffer (pH 4.8). The dialyzed enzyme solution inside the membrane was then diluted with 50 mM sodium citrate buffer (pH 4.8) to the desired concentration.

Rice bran (10 g) was mixed with 50 ml dialyzed enzyme solution (3 % w/w enzyme to rice bran) and incubated (NB-205 orbital shaking incubator, N-Biotek, UK) at 50 ºC, 150 rpm. After 2 h, the pH was adjusted to pH 8.0 by using 5 M NaOH. The solution was then mixed for further 15 min and the pH was re-adjusted, if required to pH 8.0. The slurry was homogenized in a blender for 2 min at the

maximum speed and centrifuged at 2,800 g for 20 min at 5 ºC. The creamed oil body layer floating on top of the homogenate was carefully collected and labeled as enzyme-assisted crude oil bodies (enzyme-COB).

2.4. Microscopy

2.4.1. Light microscopy

The microstructure of oil bodies was observed using a light microscope (Leitz Diaplan, Wetzlar, Germany). Images of oil bodies were obtained by using a digital camera and image processing software (PixeLink megapixel Firewire Camera, Ontario, Canada). The image scale was calibrated using a glass-mounted graticule (1 mm, 0.01 divisions: Graticules Ltd, Tonbridge, Kent, UK).

2.4.2. Transmission electron microscopy

Electron micrographs were prepared as described previously (White *et al.*, 2006). Selected areas of brown rice (approximate 1 mm thick) were fixed in 2.5 % glutaraldehyde in cacodylate buffer (0.05 M, pH 7.4) and washed with cacodylate buffer. The samples were then post-fixed in 2 % osmium tetroxide for 3 h before dehydration in increasing concentration of ethanol (50, 70, 90 and 100 %), embedding in Spurr resin and polymerization. Thin sections (0.5 µm) were cut and stained with toluidine blue for light microscopy before ultra-thin sections (80 nm) were selected, cut and mounted on

copper grids and stained using uranyl acetate and lead citrate for electron microscopy (JEOL 1010 TEM; JOEL Ltd., Herts, UK).

2.4.3. Confocal microscopy

The microstructures of lipids in oil body suspensions were examined by using a Leica SP5 confocal laser scanning microscope (Leica Microsystems, Buckingham, UK). Lipids were stained by adding 4 µl of 0.002 % w/v Nile red (Sigma, Gillingham, UK) in 100 % polyethylene glycol into 100 µl suspension. Stained suspension (8 µl) was placed on a glass slide and covered with a glass coverslip (size 18 mm × 18 mm). Nile red was excited using the 514 nm line of an Argon laser. Fluorescence intensity data were collected between 560 to 600 nm. To avoid interference due to cross fluorescence, the two emission spectra were collected using the sequential line scanning mode. Images were processed using the Leica SP5 Image Analysis software.

2.5. Compositional analysis

2.5.1. Moisture content

The moisture content was determined by drying samples (~200 mg) in opened screw-cap microtubes (2 ml) to constant weight for at least 48 h in a vacuum oven (Gallenkamp, UK) at -900 mBar and 40 ºC.

2.5.2. Soxhlet lipid extraction

Total lipids were extracted by weighing samples (5 g) into cellulose thimbles (22 mm x 80 mm, Whatman, Fisher Scientific Ltd., Loughborough). The timbers were covered with cotton wool, placed in the pre-dried extraction flask (250 ml) and connected to the Soxhlet glassware. 100 ml of n-hexane was used to extract lipids by refluxing on an electric mantle at 60 ºC at a rate of 5 - 6 drops/sec for 4 h. The extracts were dried in a rotary evaporator under vacuum. Total lipid content was determined gravimetrically and samples were stored under nitrogen (-20 ºC) in a small volume of chloroform for further analysis.

2.5.3. Bligh and Dyer lipid extraction

Lipids were recovered and purified by a modified procedure (Bligh and Dyer, 1959). Samples (0.25 g) in microtubes were extracted with solvent mixture consisting of chloroform : methanol : water = 1 : 2 : 0.8 and vortexed for 1 min. Then, chloroform (0.25 ml) was added, vortexed for 1 min and followed by water (0.25 ml) (chloroform : methanol : water = 2 : 2 : 1.8) and vortexed for 1 min. The mixture was centrifuged at 2,600 g for 10 min at room temperature to give a two-phase system. Organic bottom phase containing the lipids was recovered by using a needle syringe, evaporated under nitrogen stream to dryness and determined

gravimetrically. Samples were stored under nitrogen (-20 ºC) in a small volume of chloroform for further analysis.

2.5.4. Mini-Beadbeater (MBB) lipid extraction

The total lipid content of the dried sample (~200 mg) was determined gravimetrically by repeated extraction with isooctane (500 µl) and steel beads (1.0 mm diameter, Biospec, OK, USA) using a Mini-Beadbeater-16 (MBB) (Biospec, OK, USA) for 30 sec at the maximum shaking speed (3,450 oscillations/min). The beaten samples were centrifuged at 2,600 g for 5 min. Supernatant (450 µl) was collected in a glass Bijou bottle (7 ml). The extraction was repeated twice. The pool extracts were evaporated under nitrogen stream to dryness. The lipid content was then calculated based on dry weight basis. The samples were stored under nitrogen (-20 ºC) in a small volume of chloroform for further analysis.

2.5.5. Fatty acid composition

The lipid fraction was dried and then solubilized in chloroform (10 mg/ml). 200 µl of the lipid sample was placed into amber HPLC vials (Chromacol, Hertfordshire) and converted to fatty acid methyl esters (FAMEs) by the addition of trimethyl sulphonium hydroxide (40 µl). The solution was allowed to derivatise for 10 min at room temperature. A Trace GC Ultra gas chromatograph equipped with a DSQ mass spectrometer (Thermo Scientific, Loughborough, UK) was employed, with a flame ionization detector (FID) and auto injection

system (CTC Analytics PAL system autosampler, Essex UK) and operated in the splitless mode. One microlitre of sample was injected into a capillary column (Phenomenex Zebron ZB-FFAP, California, USA) 30 m × 0.25 mm I.D. coated with nitroterephthalic acid modified polyethylene glycol (0.25 µm film thickness). Injection temperature was 200 ºC. The oven temperature was initially held at 120 ºC for 1 min and increased to 250 ºC at a rate of 5 ºC/min for 4 min. The carrier gas was helium (30 ml/min). Retention times of standards (Supelco 37 Component FAME Mix, Supelco, PA, USA) were used to identify detected FAMEs. Methyl heptadecanoate (250 µg/ml in chloroform) was used as an internal standard. Identification was also verified by comparing mass spectrum with standard library through Thermo Scientific Xcalibur V1.4 software program.

2.5.6. Lipid profile thin layer chromatography (TLC)

20 µl of lipid fractions in chloroform (25 mg/ml) were spotted onto an Alugram® silica gel 60 aluminium sheet 20 x 20 cm (Macherey-Nagel, Germany) using a small syringe. The plate was developed in a TLC tank containing hexane : diethyl ether : acetic acid, 80 : 20: 2 by volume (Christie, 2003) and then allowed to dry under nitrogen gas. Simple lipids were visualized as brown spots in a tank of iodine vapor.

2.5.7. Protein content

The protein content of the defatted sample (as described in section 2.5.4) was measured using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). The samples were dried at 60 ºC for 30 min and then solubilized with 2 % sodium dodecyl sulfate (SDS) solution (1 ml) at 60 ºC for 30 min. Samples were vortexed (1 min) and centrifuged (2,600 g for 2 min). Supernatant was collected and diluted (to within the range of the standard curve) with 2 % SDS solution. 50 µl of the diluted samples were then mixed with 1 ml of standard working reagent (10 ml BCA solution and 200 µl 4 % cupric sulphate), incubated at 60 ºC for 30 min and measured absorbance at 562 nm against a blank containing everything but the sample. The protein standard curve was prepared using 50 - 400 µg/ml bovine serum albumin (BSA) (Sigma, USA).

2.5.8. Protein separation by gel electrophoresis

Protein concentration was determined using the bicinchoninic acid method (section 2.5.7). Proteins in dried oil body samples were then diluted to equal protein concentration with 2 % sodium dodecyl sulfate (SDS) solution. Protein samples (20 µl) were mixed with 20 µl of sample buffer (Laemmi sample buffer (Bio-Rad, UK) and 5 % β-mercaptoethanol), heated at 95 ºC for 5 min and cooled on ice. Proteins were characterized by gel electrophoresis (SDS-PAGE) using 15 % resolving gel in 4 % stacking gel (Bio-Rad Laboratories,

Hertfordshire, UK). The gel was positioned in SE 600 separation unit and suspended in running buffer (100 mM Tris, pH 8.3, 100 mM Tricine and 0.1 % SDS, (Bio-Rad, UK). Electrophoresis was run at 100 V until the dye front was at about 1 cm from the bottom of the gel. After the electrophoresis, the gel were fixed and stained with Coomassie blue (R-250) (Bio-Rad, UK) and destained with excess pure water and then 10 % methanol. The gel was imaged using a BIO-RAD GS-800 calibrated densitometer (Bio-Rad, Hercules, USA). Images were processed using PDQuest Quantity-one (Bio-Rad, Hercules, USA).

2.5.9. Total reducing sugar

A modification method of phenol-sulphuric acid method (Dubois *et al.*, 1956) was used. Sample solution (100 µl, diluted if necessary) was placed into a small glass test tube, and 1 ml 2.5 % phenol solution was added. Afterwards, 2.5 ml of concentrated sulphuric acid was added and the tube was shaken using a vortex mixer. The tube was allowed to cool down to room temperature. The mixture (200 µl) was transferred into a 96-well microtitre plate (Thermo Scientific, UK) and measured at 415 nm by using a microplate reader (model 550, Bio-Rad, UK).

2.6. Phytochemical analysis

2.6.1. Phytochemical extraction

Phytochemicals (tocochromanols, oryzanol and total phenolics) were extracted three times from dried samples (~200 mg, no steel beads) with methanol (800 µl) by agitating in Mini-Beadbeater-16 for 1 min at the maximum shaking speed (3,450 oscillations/min). The shaken samples were centrifuged at 2,600 g for 5 min at 5 ºC and supernatant was collected. The pool supernatant was filtered (0.45 µm) before analysis.

2.6.2. Measurement of tocochromanols and oryzanol

Tocochromanols and oryzanol were analyzed by using a method as described previously (Chen and Bergman, 2005). The RP-HPLC system consisted of Waters 2695 separation module (Massachusetts, USA) equipped with a Water 996 photodiode array detector and a Jasco intelligent fluorescent detector FP-920 (Essex, UK). The sample extract was injected through a Sentry guard-column (Waters, Nova-Pak C18, 4 µm, 3.9 x 20 mm, USA) and separated on a Nova-Pak C18 column (3.9 x 150 mm, 4 µm, Waters, USA). The initial mobile phase was 45 % acetonitrile, 45 % methanol, 5 % isopropanol, and 5 % of acetic acid (1%), at a flow rate of 0.8 ml/min, for 6 min. The mobile phase was changed linearly to acetonitrile: methanol : isopropanol at the ratio of 25 : 70 : 5 (v/v/v) over the next

10 min and held there for 12 min before being returned to the initial conditions. Filtered methanolic extracts (20 µl) were injected with a run time of 35 min. Detection was performed at an excitation wavelength of 298 nm and emission wavelength of 328 nm. Identification and quantification were made using calibration curves prepared from standards of α-, β-, γ-, δ-tocopherols and tocotrienols (2 - 15 µg/ml, Sigma Ltd., Gillingham, United Kingdom) and oryzanol (50 - 300 µg/ml, Tokyo Chemical Industry UK, Oxford, UK). A Waters Millenium 32 Chromatography Manager Software (version 3.0) was used for data analysis. Concentrations are expressed on a dry weight basis (mg/kg lipid in material).

2.6.3. Measurement of total phenolics

Total phenolic content (TPC) of a methanolic extract of dried samples was determined using a modified Folin-Ciocalteau method (Singleton *et al.*, 1999). 250 µl of the methanolic extracts was transferred into small eppendorf tubes. Folin-Ciocalteau reagent diluted to 50 % with water (250 µl) was added. The solution was mixed and left at room temperature for 1 min before 500 µl of saturated sodium carbonate was added. The mixture was shaken with a vortex mixer, allowed to stand at room temperature for 30 min and then measured absorbance at 750 nm. Amount of total phenolic was calculated by using a standard curve of gallic acid (5 - 25 mg/ml).

Results were expressed as gallic acid equivalents per kg of lipid in material on a dry weight basis (mg GAE/kg lipid).

2.7. Determination of antioxidant capacity

Antioxidant capacity of the methanolic extracts was determined by using the ferric reducing antioxidant potential (FRAP) assay as described previously (Benie and Strain, 1996). The FRAP reagents comprised of 3 solutions: first, 25 ml of acetate buffer (300 mM, pH 3) consisting of 1.55 g sodium acetate trihydrate, 0.94 g sodium acetate anhydrous and 8 ml glacial acetic acid made up to 500 ml with distilled water and then adjusted to pH 3; second, 2.5 ml of 2,4,6-tripyridyl-1,3,5-triazine solution (TPTZ) (10 mM TPTZ in 40 mM HCl); and third, 2.5 ml of iron (III) chloride hexahydrate (20 mM). The standard curve was prepared by using 1 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) stock solution in absolute ethanol. To perform the assay, FRAP reagent (100 µl) were added into each well in a microplate. Control (ethanol), standards and methanolic extracts (10 µl each) were then added and left at room temperature. The absorbance at 655 nm was monitored until the reaction is completed. The antioxidant capacity was presented in terms of Trolox equivalent per g sample on a dry weight basis (µmole TE/g).

2.8. Water Activity

Water activity (a_w) of rice bran was measured by using AquaLab Model Series 3 TE (AquaLab, USA).

2.9. Oil body physical characterization

2.9.1. Preparation of oil body suspension

An oil body suspension containing 2 % w/w lipids was prepared by mixing oil bodies with distilled water or phosphate-citrate buffer (a mixture of 0.1 M citric acid and 0.2 M dibasic sodium phosphate) at selected pH ranging between 2.0 - 8.0 with 1 unit interval using Wheaton tissue grinder (55 ml) and Potter-Elvehjem homogenizer (Fisher Scientific, Leicester, UK) passing through the mixture 10 times at 1,100 rpm. The pH of the oil body suspensions was adjusted to the desired pH by adding either 0.1 M HCl or NaOH; and the pH values were rechecked. All suspensions were allowed to equilibrate (24 h) prior to the morphological and physical characterization.

2.9.2. Particle size

The droplet diameters of oil body suspension were determined by using a laser diffraction particle size analyzer equipped with universal liquid module (LS 13 320 Beckman Coulter, High Wycombe, UK) and distilled water as fluid. The sizes were derived from the

Fraunhofer theories of light scattering using the polarization intensity differential scattering technology (PIDS) covering a size range from 0.04 - 2,000 µm, obscuration 4 with no sonication and a pump speed of 19 %. Measurements were performed three times for each replicate and each run length was 60 sec. The mean values were reported as the volume mean diameter D (4,3), the surface mean diameter D (3,2) and the median diameter.

2.9.3. Zeta potential

Oil body suspensions were diluted to a concentration of approximately 0.005 % lipids using distilled water or buffer solution at selected pH ranges to avoid multiple scattering effects. Zeta potential analysis was measured by using Delsa Nano particle sizer (Beckman Coulter, California, USA), which was based on electrophoretic light scattering techniques. Since the velocity of the particles is proportional to the amount of particle charges, zeta potential can be determined by measuring the velocity of the particles suspended in a liquid medium under an applied electric field.

2.9.4. Turbidity test

Stability of oil body suspension was determined as described previously (Tzen *et al.*, 1992). Oil body suspension (1.4 ml) was transferred into 1.8 ml cuvettes (Sarstedt, Numbrecht, Germany) covered with parafilm to prevent evaporation and subjected to minimal disturbance. The absorbance below the floated oil body

layer was measured (Varian Cary 50 Probe UV-Visible spectrophotometer, Cheshire, UK) at 600 nm against water blank over 6 h and 24 h of storage at room temperature. Turbidities of suspension were represented as the relative turbidity ($T / T_o = 10^A / 10^{Ao}$), where T is the turbidity at selected time, T_o is the turbidity at the start of the measurement, A is the absorbance at 600 nm at selected time and A_o is the absorbance at 600 nm at the start of the measurement respectively.

2.10. Oxidative stability

2.10.1. Lipid hydroperoxide concentration

The lipid hydroperoxides were measured according to a method of Shantha and Decker (1994) and modified by Nuchi *et al.* (2001). Lipid extracts (200 µl) were reacted with 2.8 ml of methanol/1-butanol (2:1, v/v), 15 µl of 3.94 M ammonium thiocyanate and 15 µl of 0.072 M iron (II) solution (consisting of the equal volume of 0.132 M $BaCl_2$ in 0.4 M HCl; and 0.144 M $FeSO_4.7H_2O$). Samples were left at room temperature for 20 min before measuring absorbance at 510 nm against a blank containing everything but the sample. Cumene hydroperoxide was used as a standard (0.01 - 0.5 mM) and results were expressed as mmole per kg of lipid in material based on a dry weight basis.

2.10.2. Free fatty acid determination

The non-esterified fatty acids were determined as described previously (Walde and Nastruzzi, 1991). Three stock solutions were firstly prepared: solution A, 0.1 M tris/HCl buffer (pH 9.0); solution B, 2 mM phenol red in 0.1 M tris/HCl buffer pH 9.0; and solution C, 50 mM bis (2-ethylhexyl) sodium sulfosuccinate (AOT) in isooctane. The assay solution D was then prepared by mixing solution A (0.375 ml), solution B (0.125 ml) and solution C (50 ml) together. Sample solutions were prepared from isopropanol extracts. Wet samples of rice bran or oil bodies (0.3 g) were weighed into centrifuge tubes and 1 ml of isopropanol was added. The tubes were shaken using a vortex mixer for 1 min. Then, 1 ml of isopropanol was added and the tubes were mixed for another min. The tubes were centrifuged at 2,800 g for 10 min. Supernatant was collected, evaporated under nitrogen stream and then, resuspended in 1 ml isopropanol. The measurements were performed by adding solution D into 1.8 ml cuvette and the absorbance at 560 nm were recorded. Afterwards, 30 µl of isopropanol extracts were added and mixed. The absorbances were recorded again. From each reading, the change in absorbance at 560 nm caused by 30 µl of the extracts could be determined and calculated as percentage of free fatty acids in total lipid extracts. Standard curve was made by using standard oleic acid 2.54 - 25.4 mM (≥ 99 % (GC), Sigma Ltd., Gillingham, UK).

2.10.3. Hexanal analysis

Hexanal produced from the secondary oxidation of lipids were measured by solid-phase micro extraction (SPME) and detected using a Trace GC Ultra gas chromatograph (Thermo Scientific, Loughborough, UK) equipped with a DSQ mass spectrometer (Thermo Scientific, Loughborough, UK) and an autosampler (CTC Analytics PAL system, Essex UK) (Fisk *et al.*, 2008). Rice bran (1 g) or oil body suspension (2 % lipid, 1 ml) was placed into a silicone/PTFE-capped 20 ml SPME vial (Chromacol, Hertfordshire, UK) together with 10 µl of 1,2-dichlorobenzene (100 ppm) as an internal standard. The vial was pre-incubated (3 min at 50 ºC with the agitation speed of 750 rpm) prior to extraction (20 min at 50 ºC); and desorption was achieved in 5 min at 250 ºC. Volatiles were removed from the headspace of the vial by adsorbing onto a SPME fiber assembly (50/30 µm DVB/Carboxen/PDMS StableFlex, Sigma Ltd., Gillingham, UK). Compounds were separated in a capillary column (ZB-5; Zebron, Phenomenex, Macclesfield, UK) 30 m long x 0.25 mm I.D. coated with 5%-phenyl-95%-dimethylpolysiloxane (1.0 µm film thickness). The oven temperature was held at 40 ºC for 1 min, ramped to 140 ºC at a rate of 3 ºC/min, ramped to 210 ºC at a rate of 15 ºC/min and held for 1 min. Injection temperature was 200 ºC. Identification and quantification of volatile components was verified by authentic standards and Thermo Scientific Xcalibur V1.4 software program.

2.11. Statistical analysis

All experiments were performed in triplicate. Data and graphs were processed using Microsoft Excel (2011) including averages and standard deviations. Statistical analysis was performed in SPSS version 19.0 software (IBM, Chicago, USA). Data were compared using one-way ANOVA at $P<0.05$.

3. RICE MILLING AND RICE BRAN EXTRACTS

Rice kernel consists of hull or husk (20 %), bran (8 %), embryo or germ (2 %) and starchy endosperm (70 %) (Orthoefer, 1996). The basic objectives of rice milling are to remove foreign materials, husk, bran layers and broken kernels from rice and produce white rice kernel for a variety of commercial purposes (Champagne, 2004). Rice milling consists of several steps including 1) cleaning of paddy, 2) removal of husk from rice kernels, 3) removal of bran layers from brown rice, 4) milled rice grading or removal of broken grains, and 5) blending and packaging of milled rice. This chapter discusses the removal of rice bran from brown rice and its chemical compositions and extracts.

3.1. Rice milling

Brown rice was used as the starting material in this study due to the availability of imported rice source in UK. The brown rice in this study had been stored for more than one year before milling. Upon milling, brown rice was first fed via a hopper into rice mill through a gap between two rubber rolls (Figure 3.1). Contaminated husk was removed from brown rice by aspiration. Afterwards, germ and bran layers were loosened and peeled off from brown rice by a friction whitener located in the lower part of the rice mill. Rice bran was separated by air suction and conveyed pneumatically. The bran was

then removed from the milling chamber through a cyclone and collected in a plastic bag. Milled rice obtained from the friction whitening was further passed through the screen separator to separate small broken rice from whole grain white rice. The amount of each rice fraction from brown rice milling is shown in Table 3.1. Milling degree was calculated based on the amount of rice bran removed from brown rice and was 9.1 ± 1.2%.

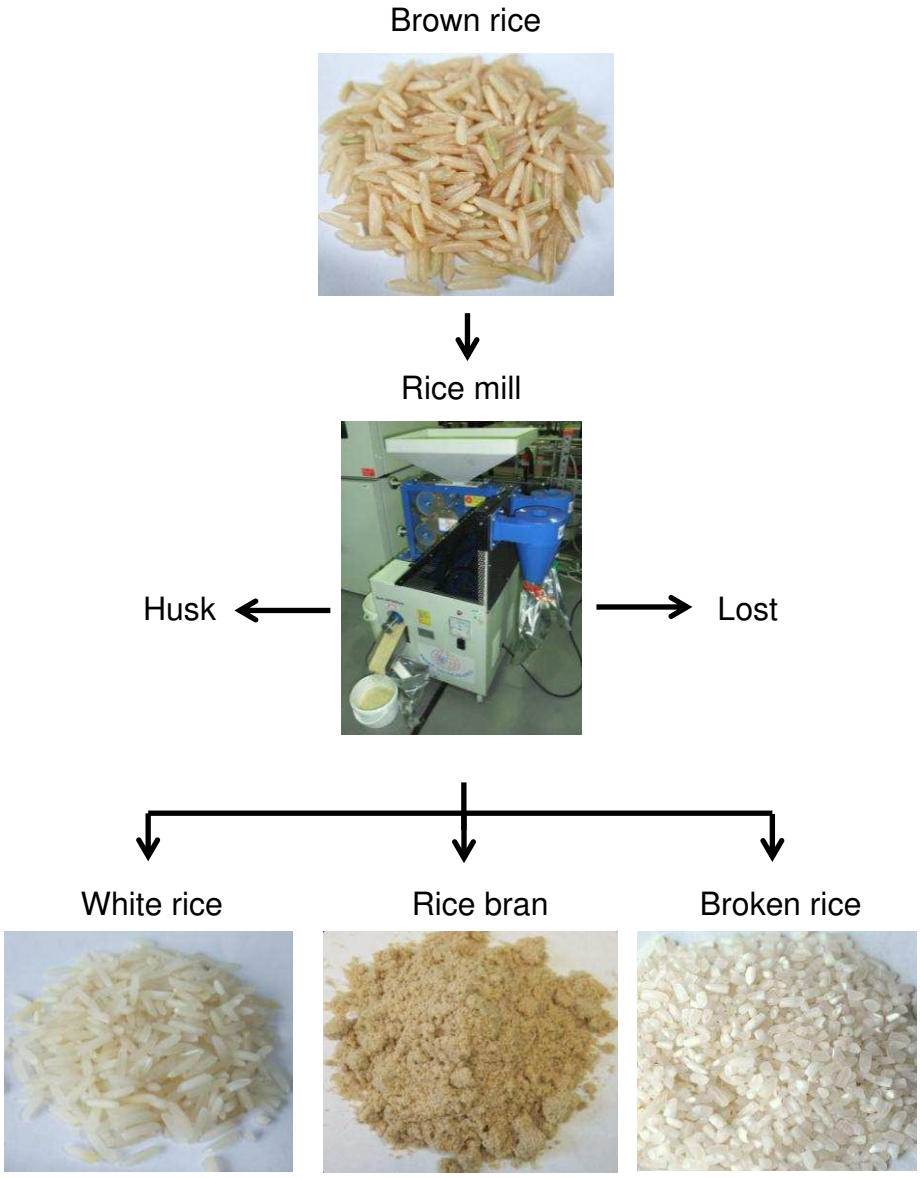


Figure 3.1 Brown rice milling

Table 3.1 Content of rice fraction from rice milling

Rice fraction	Content (% of brown rice)
White rice	81.8 ± 5.2
Rice bran	9.1 ± 1.2
Broken rice	5.6 ± 4.0
Husk	0.3 ± 0.2
Lost	3.2 ± 2.2

3.2. Chemical composition

3.2.1. Basic composition

Rice bran contained lower moisture content (9.6 ± 0.1 %) than brown rice (11.9 ± 0.1 %) and white rice (12.1 ± 0.6 %). The composition of three rice milling fractions (brown rice, white rice and rice bran) is shown in Table 3.2. Fat content was significantly ($P<0.05$) different among the rice fractions. Rice bran fraction contained noticeably higher lipid (21.1 ± 0.6 %) and protein content (15.0 ± 0.7 %) than brown rice and white rice. Approximately 59% of lipids and 16% of protein in brown rice were distributed in rice bran. Therefore, rice bran, a by-product from rice milling, was selected for oil body recovery, which was presented further in this report.

The lipid and protein concentrations in each rice fraction were similar to the typical values in literature (Marshall and Wadsworth, 1994). The composition of rice fractions varied according to rice

varieties, environmental conditions, growing locations, processing factors and storage.

Table 3.2 Lipid and protein content (% dry weight basis) of rice fractions

Rice fraction	Lipid content (%)	Protein content (%)
Brown rice	2.5 ± 0.1	8.7 ± 0.6
White rice	0.5 ± 0.1	8.4 ± 0.7
Rice bran	21.1 ± 0.6	15.0 ± 0.7

3.2.2. Phytochemical composition

The effects of solvent types (methanol, hexane, ethanol, isopropanol, isooctane and dichloromethane), solvent to bran ratios (1:1, 2:1 and 4:1 w/v) and methods of mixing (vortex mixing and beating by MBB) on the extraction of tocochromanols and oryzanol were first studied. Methanolic extracts of rice bran were enriched in tocochromanols and oryzanol compared with the other extraction solvents (Figure 3.2). Our results agree with literature; the study of Chen and Bergman (2005) concluded that methanol was a superior extraction solvent relative to hexane at ambient temperature. The hydroxyl groups on the structure of tocochromanols and oryzanol make these phytochemicals more extractable in methanol than in hexane. The concentration of phytochemicals increased with increased solvent to bran ratio and the degree of mixing (violently agitation of MBB compared to vortex mixing). Therefore,

tocochromanols and oryzanol were extracted over the course of this study using methanol (solvent to bran ratio of 4:1 w/v), and agitating with a Mini-Beadbeater (MBB).

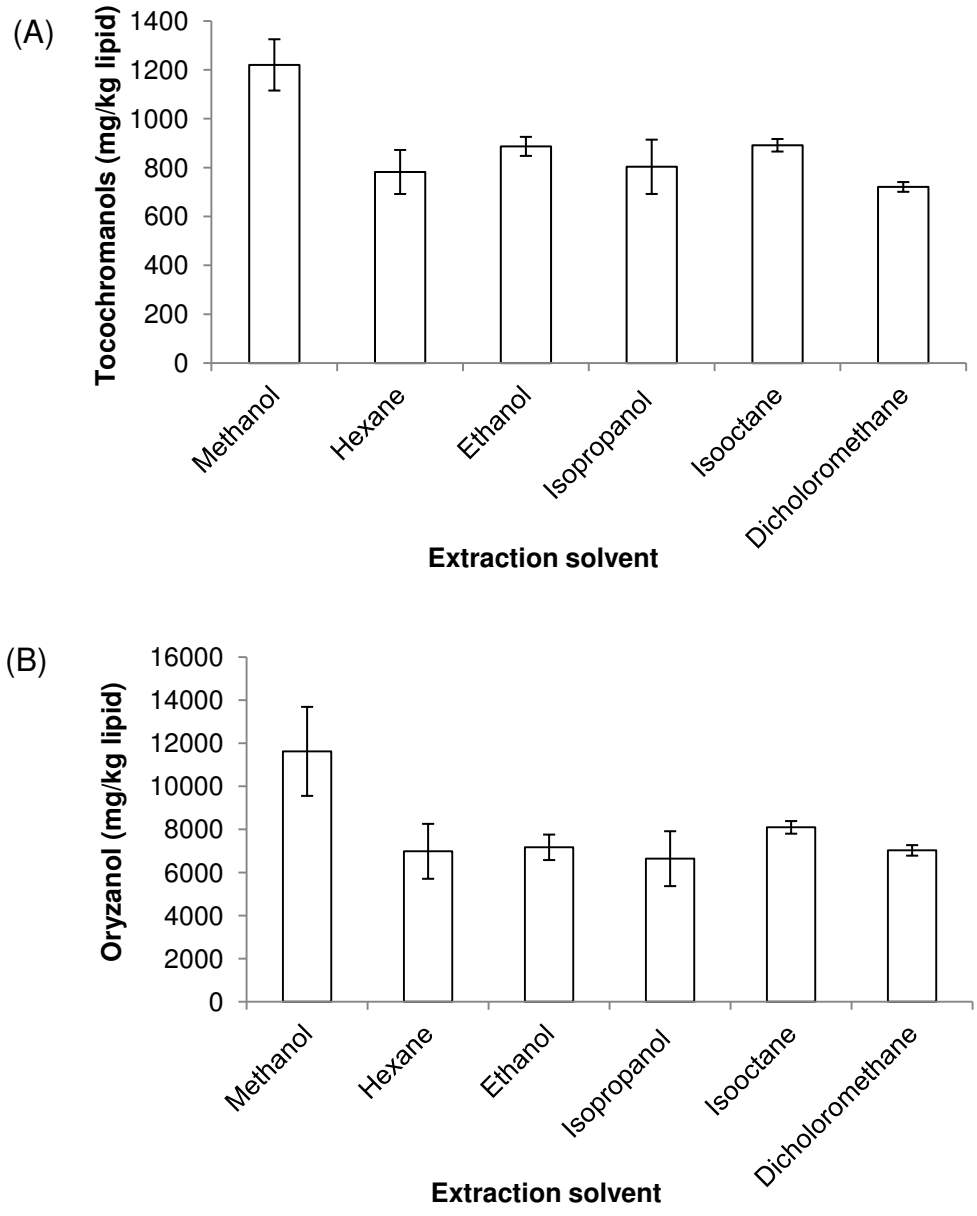


Figure 3.2 Phytochemical concentrations in rice bran extracted by different solvent (A) tocochromanols and (B) oryzanol

Methods of phytochemical detection were examined to develop optimum separation conditions of tocochromanols from oryzanol in the same sample run. The use of UV absorption in tocochromanol detection resulted in poor separation of each isoform. The use of fluorescence detection, however, increased the resolution of each tocochromanol. The HPLC chromatograms of standard mixtures of tocotrienols (T3s) and tocopherols (Ts) and methanolic extracts of rice bran (Figure 3.3) show that a group of tocotrienol isoforms (retention time of 6.2 - 8.6 min) eluted before that of tocopherols (retention time of 13.2 - 16.5 min). The elution order of tocochromanol isoforms was δ-, then the coelution of β- and γ- and finally α-isoform. Chromatographically, coelution of β- and γ-isoforms is common in reverse-phase HPLC, while normal-phase HPLC shows a clear separation of all eight isoforms (chromatogram not shown). Despite the drawback of RP-HPLC in tocochromanol separation, it provides the simultaneous detection of both tocochromanols and oryzanol in a single run.

A standard mixture of oryzanol components diluted in methanol at various concentrations (5 - 30 mg/L) was scanned from 230 to 400 nm for the maximum absorbance using spectrophotometric method (Figure 3.4). The UV absorption spectra show three major peaks at 240, 300 and 325 nm, whilst the maximum absorbance is at 325 nm. Similar absorption spectrum

profiles were published previously (Diack and Saska, 1994; Tanaka *et al.*, 1971).

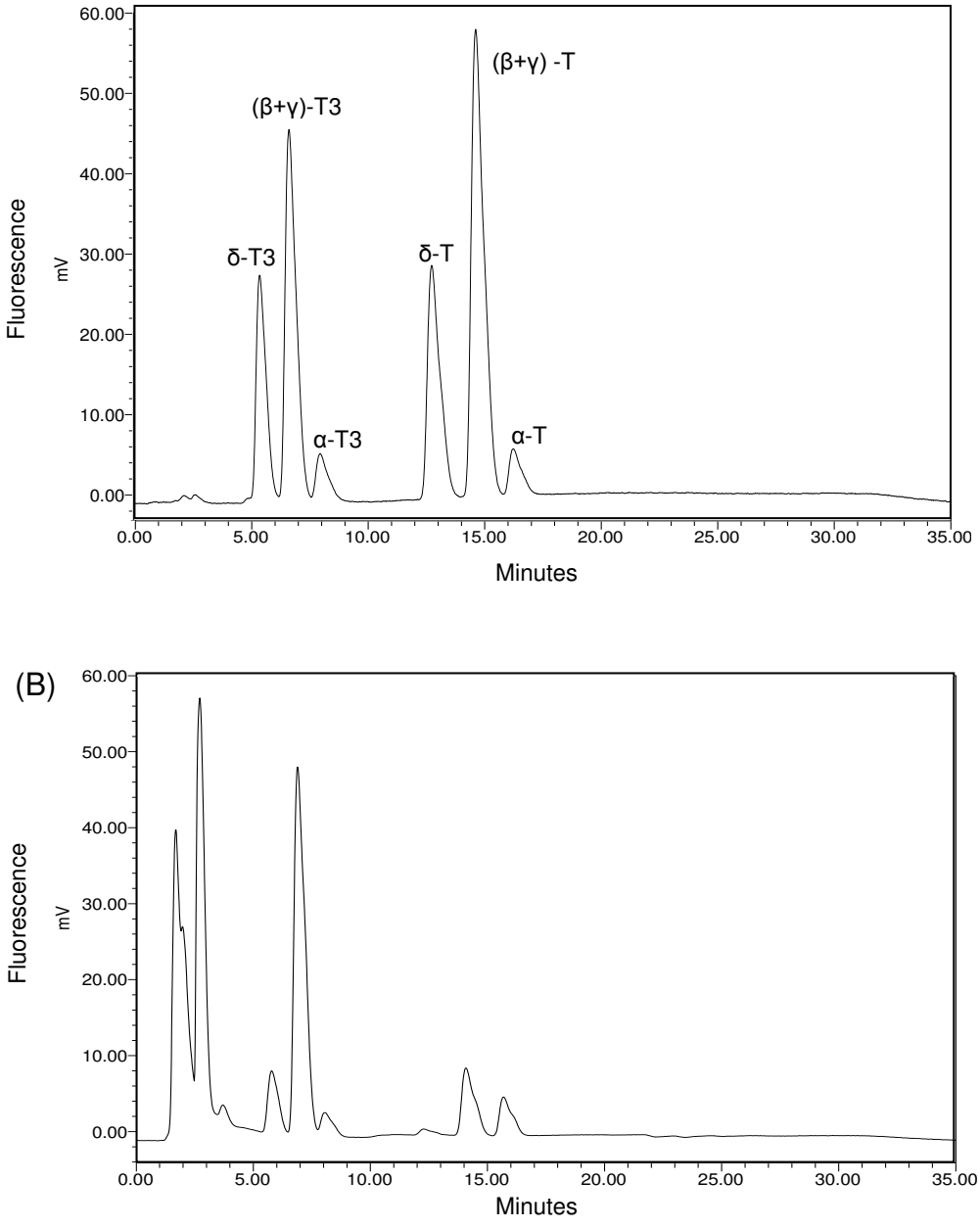


Figure 3.3 Chromatograms of the RP-HPLC of (A) standard mixtures of tocotrienols (T3s) and tocopherols (Ts) and (B) methanolic extracts of rice bran.

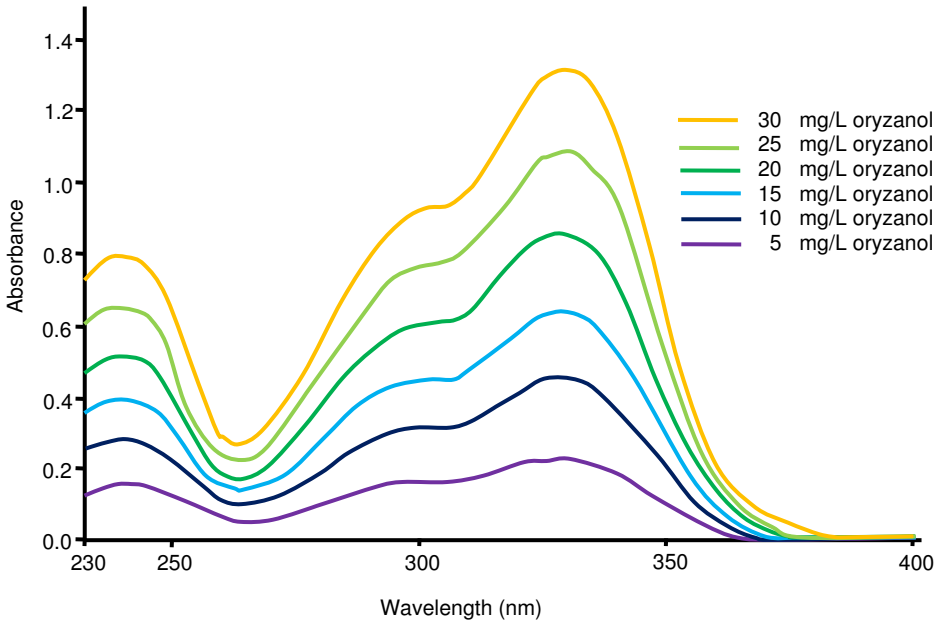


Figure 3.4 UV absorbance of standard mixture of oryzanol scanned from 230 - 400 nm

From the above spectra data, absorbance at 325 nm was used as a measure of oryzanol fraction after RP-HPLC via photodiode array (PDA) detector (Figure 3.5). However, individual ferulates in oryzanol could not be distinguished in this study because of the lack of standards of individual oryzanol component. In addition, the chromatograms of both tocochromanols and oryzanol were similar to those published previously (Chen and Bergman, 2005; Rogers *et al.*, 1993). Oryzanol was previously identified as a mixture of 10 components (Xu and Godber, 1999). They reported that cycloartenyl ferulate, 24-methylene cycloartanyl ferulate and campesteryl ferulate were the three main ferulates composing oryzanol in rice bran oil.

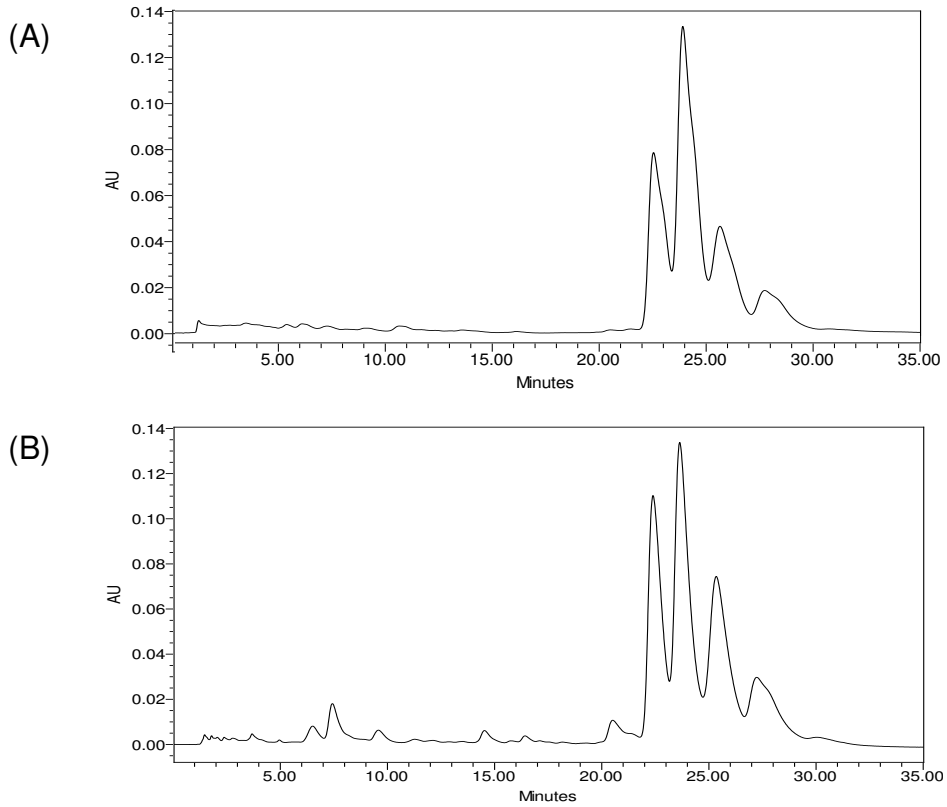


Figure 3.5 Chromatograms of the RP-HPLC of (A) standard mixture of oryzanol and (B) methanolic extracts of rice bran.

The tocochromanol and oryzanol concentrations were quantified against a standard curve (2 - 15 mg/L tocochromanols and 50 - 300 mg/L oryzanol) (Figure 3.6 and Figure 3.7 respectively). High correlation coefficient values (R^2 > 0.99) of each standard indicated a linear response over these concentration ranges. Therefore, RP-HPLC was chosen as a method to determine phytochemical composition in rice bran in this study. Tocochromanols were quantified by fluorescence detection while oryzanol was by photodiode array detection.

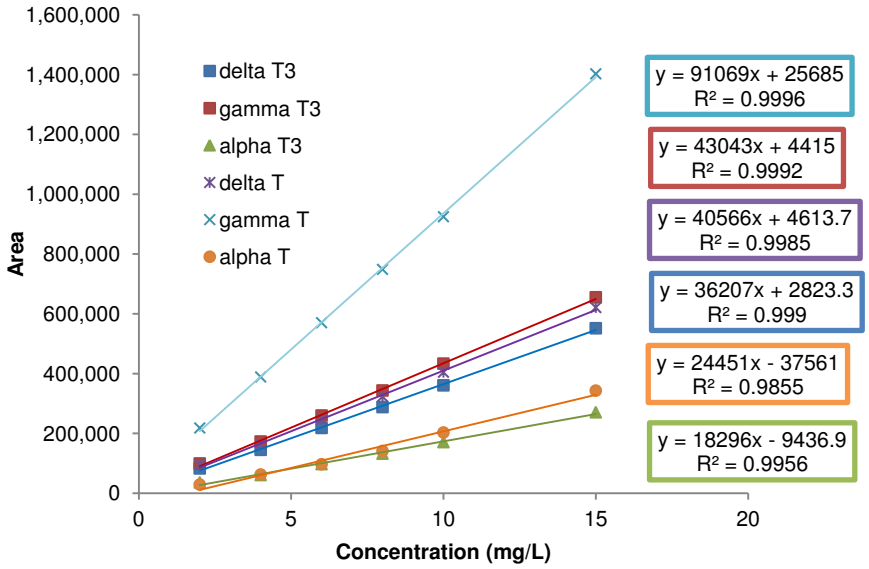


Figure 3.6 Standard curve of tocochromanols determined from RP-HPLC

α-, γ- and δ- tocotrienols (T3) and tocopherols (T) (2 - 15 mg/L in methanol)

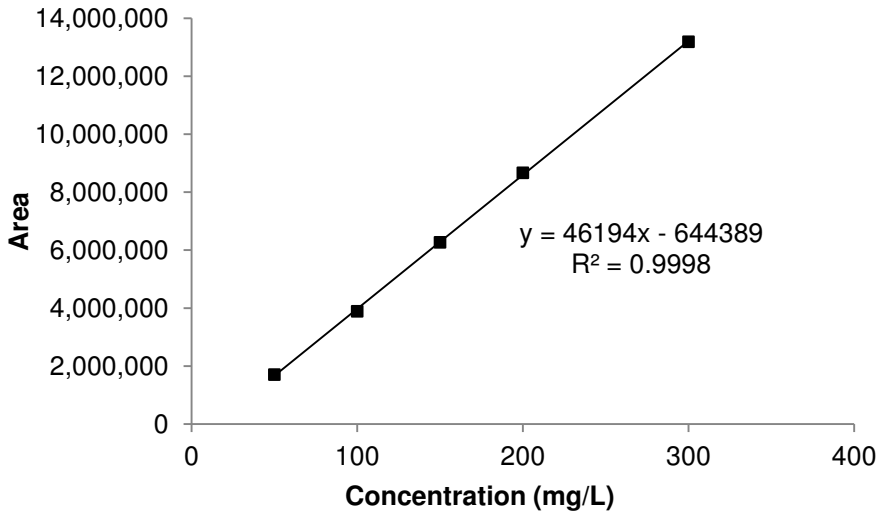


Figure 3.7 Standard curve of oryzanol determine from RP-HPLC

The quantity of tocotrienols and tocopherols (total tocochromanols) varied amongst the rice fractions (Table 3.3). The values are shown based on the amount in mg per unit mass of dry materials. The values based on the amount in mg per unit mass of lipid are also given in order to follow the fate of these phytochemicals in the lipid fraction through oil body extraction in the next chapter. Note that high values of these phytochemicals per unit mass of lipid in white rice and brown rice were because of the low lipid content in these fractions. The total tocochromanols and oryzanol were most concentrated in the rice bran fraction (233 ± 4 mg/kg dry mass), followed by brown rice (33 ± 1 mg/kg) and white rice (4.9 ± 0.3 mg/kg) respectively. Tocotrienols were the most abundant form of tocochromanols in all rice fractions: rice bran (72.7 %), white rice (97.6 %) and brown rice (79.3 %). The major tocochromanol isoform in all rice fractions was γ-tocotrienol. The order of each tocochromanol level in rice bran was γ-T3 (53.9 %) > α-T (19.4 %) > δ-T3 (10.7 %) > α-T3 (8.1 %) > γ-T (7.2 %) > δ-T (0.7 %). Although α-tocopherol has been historically considered to have the greatest biological activity among the tocochromanol isoforms, tocotrienols has received more attention from researchers recently. This due to the beneficial properties of α- and γ-tocotrienols such as inhibiting cholesterol synthesis, lowering levels of serum cholesterol (Qureshi *et al.*, 2001), free radical scavenging activity (Serbinova *et al.*, 1991) and the abundance of γ-tocotrienols in rice bran.

Total tocochromanol level in rice bran in this study was in the range (179 - 389 mg/kg) found in studies of U.S. rice cultivars (Bergman and Xu, 2003). Rice bran from a long grain variety (Tebonnet, Newbonnet and Lemont) contained 300 mg/kg total tocochromanols (Shin *et al.*, 1997). Chen and Bergman (2005) reported levels of 109 - 322 mg tocochromanols/kg rice bran fresh weight from U.S. long and medium grain variety. Higher content of total tocochromanols (1,360 mg/kg dry weight) in *Japonica* rice bran was reported by Sookwong *et al.* (2007). Venezuelan brown rice was reported to contain tocochromanols 46.4 - 65.0 mg/kg (Aguilar-Garcia *et al.*, 2007). Heinemann et al. (2008) who have found the total tocochromanol values in brown rice can vary from 10.4 - 32.5 mg/kg according to rice origins.

The content of oryzanol measured in this study also varied widely amongst rice fractions. The bran fraction showed the highest oryzanol content (2,312 ± 40 mg/kg dry mass) of all fractions, followed by brown rice (320 ± 12 mg/kg) and white rice (33 ± 2 mg/kg) respectively. The concentrations of oryzanol in brown rice were similar to the values reported by Miller and Engel (2006) (260 - 630 mg/kg) and Aguilar-Garcia *et al.* (2007) (200 - 390 mg/kg). However, lower levels of oryzanol in brown rice have been reported; 130 - 290 mg/kg (Heinemann et al., 2008). The concentrations of oryzanol in rice bran have been reported to be 3,400 - 4,200 mg/kg

bran fresh weight (Chen & Bergman, 2005), 1,550 - 2,720 mg/kg bran dry weight (Aguilar-Garcia et al., 2007) and 2,510 - 6,864 mg/kg (Bergman and Xu, 2003). Hexane and isopropanol, instead of methanol, have also been used as an extraction solvent for oryzanol, giving values of 2,450 - 2,930 mg/kg in extrusion stabilized rice bran (Hu *et al.*, 1996).

Differences in the levels of phytochemicals in rice might be attributed to the rice cultivar, growing location, degree of milling, method of extraction and the amount of bran removed by polishing (Aguilar-Garcia *et al.*, 2007; Chen and Bergman, 2005; Heinemann *et al.*, 2008; Lloyd *et al.*, 2000).

Table 3.3 Phytochemical composition of different rice fractions[1]

Phytochemical[2]	Rice bran		White rice		Brown rice	
	mg/kg mass	mg/kg lipid	mg/kg mass	mg/kg lipid	mg/kg mass	mg/kg lipid
δ-T3	25 ± 1	153 ± 5	1.3 ± 0.2	240 ± 30	4.2 ± 0.2	167 ± 7
γ-T3	126 ± 3	771 ± 18	3.5 ± 0.2	641 ± 37	20 ± 1	821 ± 18
α-T3	19 ± 2	116 ± 10	ND[3]	ND	1.5 ± 0.1	61 ± 6
δ-T	1.5 ± 0.1	9.4 ± 0.6	ND	ND	0.7 ± 0.2	29 ± 8
γ-T	17 ± 1	104 ± 5	0.1 ± 0.0	21 ± 3	2.0 ± 0.1	80 ± 4
α-T	45 ± 2	277 ± 15	ND	ND	4.1 ± 0.4	164 ± 17
Total T3	170 ± 4	1,040 ± 22	4.8 ± 0.3	881 ± 47	26 ± 1	1,049 ± 20
Total T	64 ± 3	390 ± 16	0.1 ± 0.0	21 ± 3	6.8 ± 0.5	273 ± 19
Total	233 ± 4	1,430 ± 27	4.9 ± 0.3	902 ± 47	33 ± 1	1,322 ± 28
Oryzanol	2,312 ± 40	14,174 ± 245	33 ± 2	5,953 ± 345	320 ± 12	12,788 ± 486

[1] Results are expressed as mg/kg dry mass of rice fraction and mg/kg lipid in rice fraction on a dry weight basis (n = 3, ± SD)
[2] T3 = tocotrienol; T = tocopherol; Total = total tocochromanols (T3+T)
[3] ND = Not detected

3.3. Rice bran chemical extracts

3.3.1. Lipid extracts

It is claimed that accurate quantification of lipid content in cereal grain is difficult due to the large amount of lipid present inside the cells and starch granule. This makes the lipid inaccessible to extraction solvent under normal conditions. The complete removal of lipid can be achieved by solvent extraction followed by acid hydrolysis of the extracted residue (Inkpen and Quakkenbush, 1969). It was found that solvents systems extracted 0.96 - 1.4 % lipid from wheat flour, but subsequent acid hydrolysis removed 0.15 - 0.28 % additional lipid. However, the hydrolytic process may cause an increase in fatty acids and a decrease in oryzanol concentration in rice bran oil (Orthoefer, 1996). Therefore, for this work rice bran lipid content was measured using different types of techniques (Soxhlet (see section 2.5.2), Bligh and Dyer (section 2.5.3) and Mini-Beadbeater (MBB) (section 2.5.4)) and extraction solvents (isooctane, hexane, and diethyl ether) to evaluate the lipid extraction protocol.

The conventional method for lipid extraction by Soxhlet apparatus with hexane was the most efficient method, but only by a small margin as it extracted the highest lipid content ($P<0.05$) by the repeated extraction with clean and warm solvent at longer time as compared with MBB and Bligh and Dyer method (Figure 3.8).

However, there are disadvantages of Soxhlet procedure; it is time consuming; large volume of solvent required; and boiling solvent is hazardous for routine use with a large number of samples. There was no significant difference in lipid yield between MBB and Bligh and Dyer methods. Nevertheless, the Bligh and Dyer method is a relatively involved method (Bligh and Dyer, 1959). Therefore, an alternative and more rapid method of MBB for total lipid extraction was developed. MBB disrupts tough plant cells like rice bran through the action of, in this case, small metal balls (1.0 mm in diameter) being vigorously shaken in the presence of solvent. After centrifugation and removal of solvent, lipid content was determined gravimetrically. MBB method is suitable for routine analysis and requires only small amount of samples (~ 0.2 g) with short analysis time.

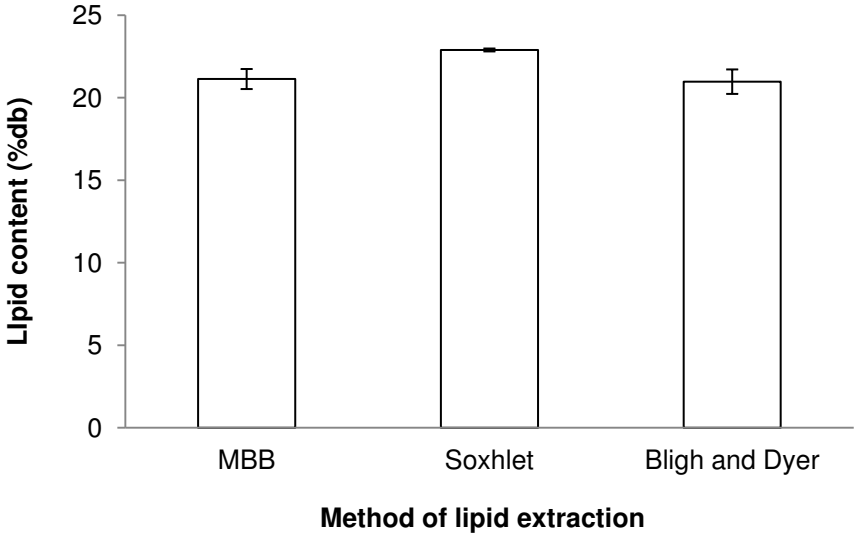


Figure 3.8 Lipid content of rice bran determined by different methods of extraction

Mini-Beadbeater (MBB)

Lipid content of dried rice bran extracted using MBB with isooctane as the extraction solvent (21.1 ± 0.6 %) was statistically similar ($P<0.05$) to that of hexane (20.6 ± 1.0 %), but was significantly lower than that of diethyl ether (27.1 ± 0.4 %). The high weight of extracted lipids from diethyl ether may be caused by the partial carried over of water-soluble components such as hexoses. In addition, diethyl ether is highly flammable with the autoignition temperature of 160 ºC, therefore, it can be ignited during solvent removal on a hot plate. Because of the slightly higher lipid content obtained from isooctane compared with hexane, isooctane was then used to extract lipids with MBB from rice bran.

3.3.2. Lipid classes

Lipid extracts from rice bran were further separated by thin layer chromatography (TLC) and stained using iodine vapor for the analysis of lipid classes. TLC separation of lipid classes in commercial refined rice bran oil and fresh rice bran lipids extracted by different methods is shown in Figure 3.9. The lipid extracts were separated and identified by the retention factor (Rf) according to the literature (Christie, 2003). The separated lipids included sterol esters, triacylglycerols, free fatty acids, diacylglycerols, monoacylglycerols and phospholipids at the origin. Undoubtedly, the major lipid content of commercial rice bran oil was triacylglycerols and a very small amount of free fatty acids were found. In contrast, the major

component of the lipids extracted from fresh rice bran, either by MBB or Bligh and Dyer extraction, was free fatty acids. The high free fatty acid concentration in the freshly milled rice bran lipids may be explained by the age of the brown rice (more than 1 year old) from which the bran was derived in this study.

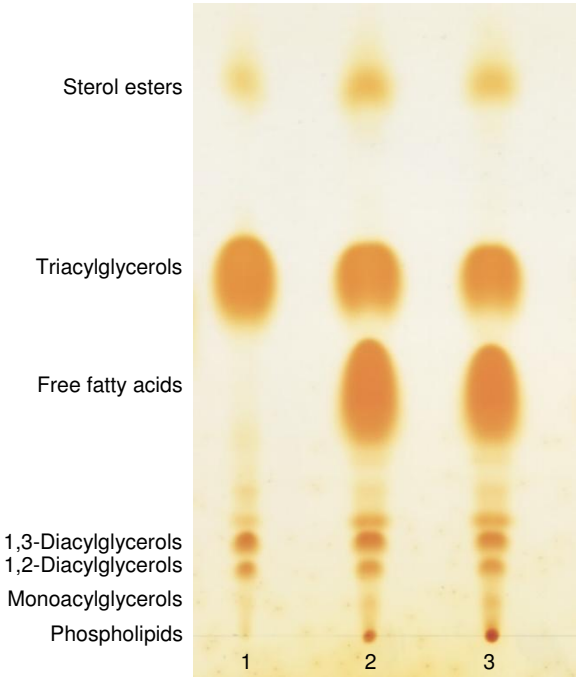


Figure 3.9 Separation of rice bran lipid classes on TLC plate (Silica Gel 60)

Developing solvent: hexane-diethyl ether-acetic acid (80:20:2 by volume). 1, commercial rice bran oil; 2, lipid extracts from Mini-Beadbeater (MBB) method and 3, lipid extracts from Bligh and Dyer method

3.3.3. Fatty acid composition

Figure 3.10 shows fatty acid composition of extracted lipid from rice bran. There was no difference ($P<0.05$) among the fatty acid compositions of rice bran lipid extracts from different methods. Most of the fatty acid composition determined is similar to that of literature (McCaskill and Zhang, 1999; Hoed et al, 2006). Oleic acid (48.0 %) is the major fatty acid, followed by linoleic acid (28.4 %), palmitic acid (19.9 %), stearic acid (2.5 %) and linolenic acid (1.3 %) respectively. It can be seen that unsaturated fatty acids, oleic acid and linoleic acid, constitute more than 70% of the fatty acids of the rice bran glycerides.

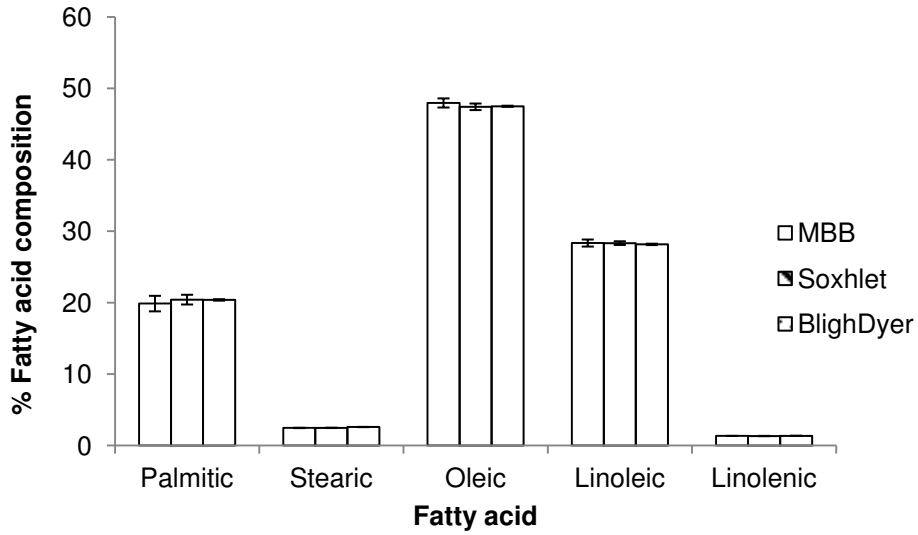


Figure 3.10 Fatty acid composition of rice bran lipids from different methods of extraction

Mini-Beadbeater (MBB)

3.3.4. Pigment extracts

The absorption spectrum of the rice bran methanolic extract indicates a major peak at around 410 and 670 nm (Figure 3.11 A). This may be due to the presence of chlorophyll in the methanolic extracts. The absorption spectra of spinach methanolic extract support this observation (Figure 3.11 B). Generally, chlorophyll is green because it absorbs light at 430 nm (blue) and 680 nm (red) of the visible spectrum (Buchanan *et al.*, 2000). The absorbance in the region of 400 - 500 nm may show the presence of other pigments in the extracts. However, no further experiments on pigment extracts were performed. Pigments in crude rice bran oil were reported as carotenes, chlorophyll and protein degradation products (Orthoefer, 2005).

In addition, diethyl ether gave a richer color of extracts from rice bran (Figure 3.12). The non-polar nature of diethyl ether may contribute to the solvation of some lipophilic pigments and the partial carryover of water by diethyl ether. These limited the ability of diethyl ether for using as the extraction solvent.

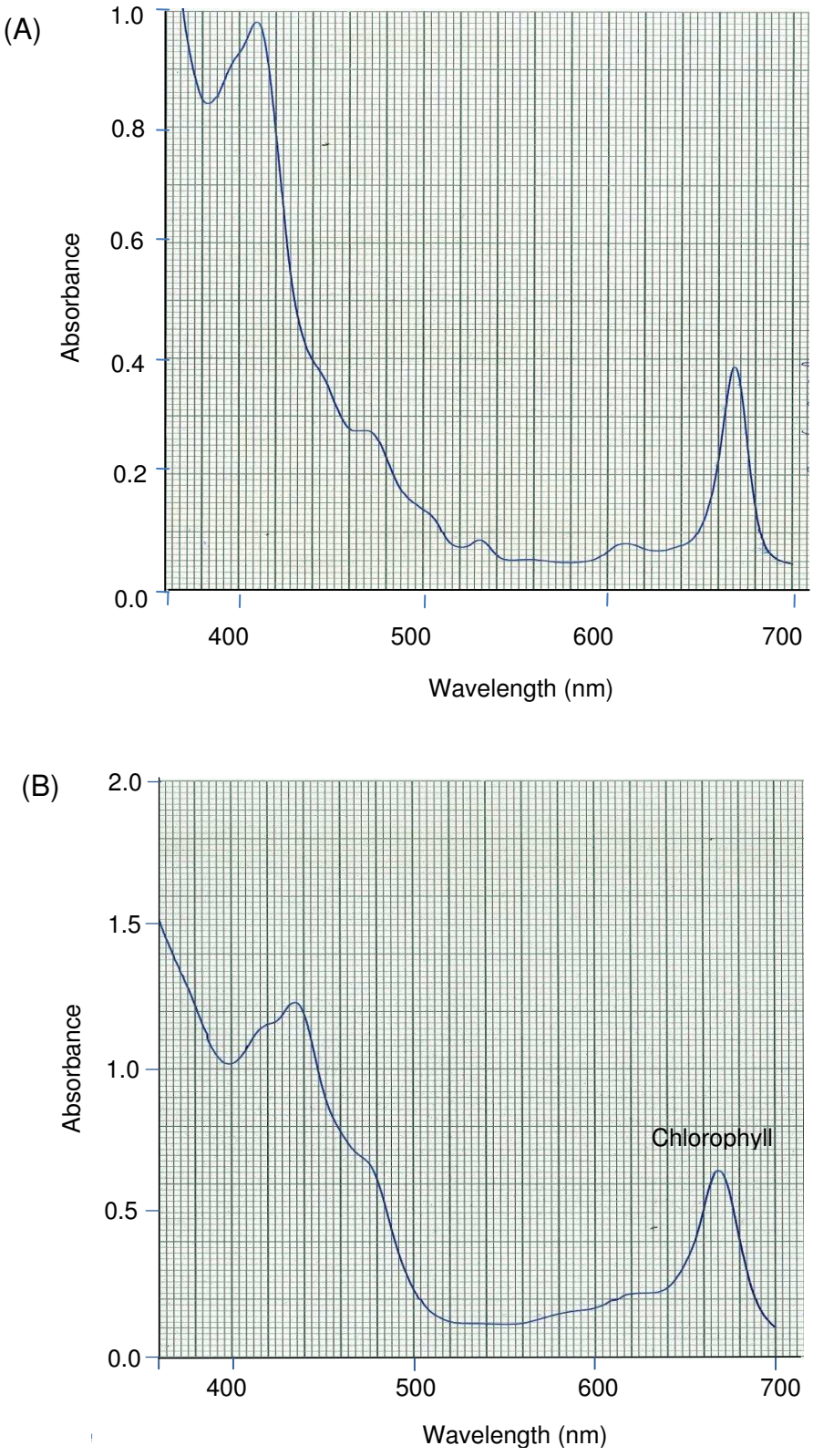


Figure 3.11 Chromatograms of methanolic extracts from (A) rice bran and (B) spinach leaves

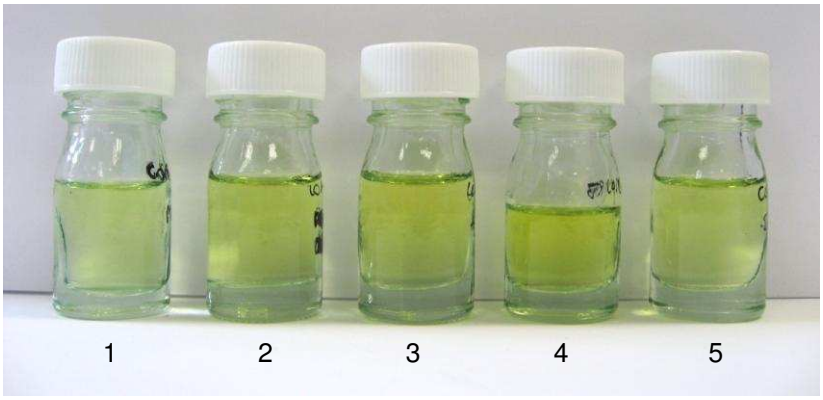


Figure 3.12 Rice bran extracts from various extraction solvents
1, methanol; 2, ethanol; 3, butanol; 4, diethyl ether; and 5, isooctane

3.4. Summary of results

Brown rice was milled on site in order to produce freshly milled rice bran for the present study. Brown rice milling yielded white rice and broken rice; rice bran, germ and husk were by-products from the rice milling. Rice bran contained noticeably higher lipid and protein content than brown rice and white rice. Therefore, rice bran was selected for oil body recovery, which was presented further in this report.

A method that could simultaneously measure the concentration of total tocochromanols (tocotrienols and tocopherols) and oryzanol was established. The optimal conditions for tocochromanols and oryzanol extraction were with methanol at 4:1 solvent to bran ratio and agitating with a Mini-Beadbeater (MBB).

RP-HPLC was chosen as a method to determine the phytochemical composition in rice bran. Tocochromanols were quantified by fluorescence detection while oryzanol was quantified by photodiode array detector. High correlation coefficient values ($R^2 > 0.99$) for each standard was obtained. The total tocochromanols and oryzanol were most concentrated in the rice bran fraction (233 ± 4 mg/kg and 2,312 ± 40 mg/kg respectively) followed by brown rice (33 ± 1 mg/kg and 320 ± 12 mg/kg respectively) and white rice (4.9 ± 0.3 mg/kg and 33 ± 2 mg/kg respectively). The major tocochromanol isoform in all rice fractions was γ-tocotrienol.

Lipid extraction protocol was evaluated. Mini-Beadbeater (MBB), Soxhlet lipid extraction and Bligh and Dyer method were compared. MBB is an alternative and more rapid method for the total lipid extraction. It was suitable for routine analysis and required only a small amount of samples. Isooctane is a promising alternative solvent to extract total lipids from rice bran.

TLC separation of rice bran lipid classes revealed that the major lipid class of commercial rice bran oil was triacylglycerols. In contrast, the major component of the lipids extracted from rice bran in this study was free fatty acids and it is suggested that this was because of the age of the brown rice from which the bran was derived.

Rice bran lipids were composed of oleic acid (48.0 %), linoleic acid (28.4 %), palmitic acid (19.9 %), stearic acid (2.5 %) and linolenic acid (1.3 %). In addition, the absorption spectrum of the methanolic extract showed a major peak at around 410 and 670 nm. This may indicate the presence of chlorophyll in the rice bran methanolic extract.

4. RICE BRAN OIL BODY CHARACTERIZATION

Rice bran, a by-product from rice milling, composed of pericarp, seed coat, nucellus, aleurone, germ and some endosperm (Champagne, 2004). Rice bran is a major source of rice lipids, which contribute to nutritional, sensory and functional properties. Additionally, minor constituents in rice bran lipids such as tocochromanols and oryzanol have antioxidant activity that may reduce the risk of certain chronic diseases that are promoted by reactive oxygen species and free radicals (Chotimarkorn *et al.*, 2008; Xu *et al.*, 2001; Yoshida *et al.*, 2003).

Lipids accumulated in rice bran appear as small spherical droplets called oil bodies. Oil body recovery is an alternative approach to recover lipids and other lipophilic phytochemicals such as tocotrienols and tocopherols. Despite attempts to extract oil bodies from rice bran (Tzen *et al.*, 1990; Tzen and Huang, 1992), the characteristics and phytochemical content of rice bran oil bodies *in vitro* have not been studied. This chapter discusses the recovery of rice bran oil bodies by various methods and characterization; with a focus on chemical composition, phytochemical composition and physical properties that contribute to the stability and functionality of the prepared suspension.

4.1. Rice bran oil body recovery

As described in Chapter 3, rice bran is richer in both protein and lipids than brown rice and white rice. Oil bodies from whole-grain basmati brown rice and white rice were hardly recovered and the yield proved extremely low (data not shown). Thus, these sources were judged to be too inefficient for commercial application. The focus of this investigation was therefore on oil bodies recovered from the bran only. The fresh rice bran in this study derived from brown rice grains that had been stored for more than one year. In order to obtain small, intact and stable oil bodies enriched in oil for further studies, different methods were employed to recover oil bodies from rice bran. These include 1) mortar and pestle method, 2) water-based method, 3) alkali-based method, and 4) enzyme-assisted method.

4.1.1. Mortar and pestle oil body recovery

The first attempt to recover oil bodies was done by grinding rice bran using a mortar and pestle (see section 2.3.1). Pre-soaked rice bran (50 g in 250 ml distilled water) was ground for 5 min. After filtration and centrifugation, the creamed oil body layer floating on the top of the homogenate was carefully collected and labeled as mortar and pestle-crude oil bodies (MP-COB). The recovered oil bodies contained lipids and protein 82.4 ± 2.6 % and 8.0 ± 0.4 % respectively. However, the volume mean diameter of oil body droplet

or D(4,3) (as in section 2.9.2) was large (5.8 ± 0.1 μm) and free oils (as stained in pink by Oil Red O) were observed under light microscope (Figure 4.1). The yield of lipid recovery, as determined by the lipids found in oil bodies relative to the lipids in parental rice bran, was relatively low (14.6 ± 1.6 %, Table 4.1). Since the grinding was performed by hand, the homogenization was slow, tedious and uncontrollable. Therefore, other methods for oil body recovery were purposed.

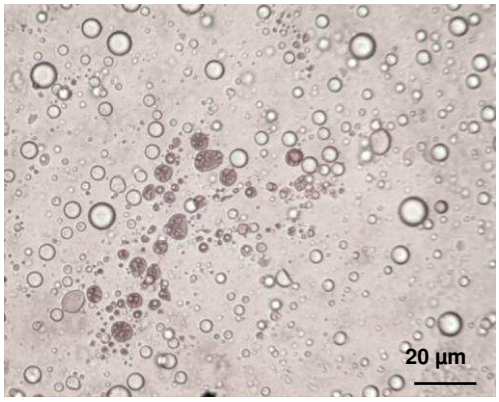


Figure 4.1 Light micrograph of rice bran oil bodies recovered by mortar and pestle method. Free lipids stained in pink by Oil Red O.

4.1.2. Water-based oil body recovery

The water-based oil bodies were recovered according to section 2.3.2. Pre-soaked rice bran (50 g in 250 ml distilled water) was homogenized in a kitchen blender for 2 min at the maximum speed. After filtration and centrifugation, the creamed oil body layer floating on the top of the homogenate was carefully collected and labeled as crude oil bodies (COB). For purification, the crude oil

bodies were either washed with water and labeled as water-washed oil bodies (WWOB) or with 9 M urea and labeled as urea-washed oil bodies (UWOB). By changing the method of milling from a mortar and pestle to a mechanical blender, the yield of lipids in recovered crude oil body was statistically increased (30.0 ± 0.4 %, Table 4.1). However, after the purification, the oil droplets in both water-washed and urea-washed oil body preparations were much larger in size (10 - 12 µm) than the size of the oil droplets in the crude oil body preparation and some irregularly shaped droplets were observed (Figure 4.2).

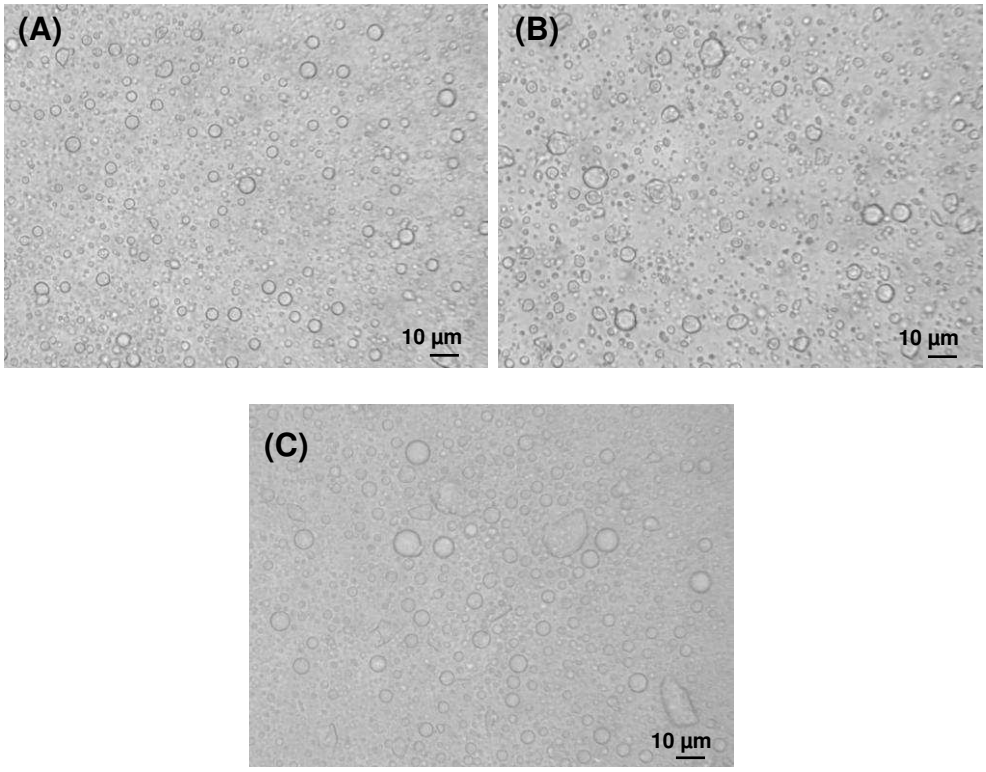


Figure 4.2 Light microscope images of rice bran oil body preparations

A, crude oil bodies; B, water-washed oil bodies; C, urea-washed oil bodies

4.1.3. Alkali-based oil body recovery

In order to produce purified intact and undamaged oil bodies from rice bran for further studies, a modified method of Beisson *et al.* (2001) was used (see section 2.3.3). Rice bran (50 g) was homogenized in 250 ml 0.1 M Tris-HCl pH 8, containing 1 mM EDTA by using a Silverson high shear laboratory mixer with Emulsor screen at 6,000 rpm for 40 sec. After filtration and centrifugation, the creamed oil body layer floating on top of the homogenate was carefully collected and labeled as alkali-crude oil bodies (alkali-COB). The crude oil bodies were further purified by washing with 0.1 M $NaHCO_3$, containing 1 mM EDTA and labeled as alkali-washed oil bodies (alkali-WOB).

The lipid recovery yield of alkali-COB (28.9 ± 2.8 %, Table 4.1) was statistically similar to that of COB from the water-based method, but significantly higher than that of the mortar and pestle method. However, the oil body purification by alkali washing protocol resulted in the very low lipid recovery yield (2.8 ± 0.3 %, Table 4.1). The purification removed the defected oil bodies, leaving behind small and intact oil bodies (diameter of 2 - 3 μm) as can be seen by light micrographs (Figure 4.3). The alkali-WOB also remained as discrete organelles and did not aggregate or coalesce.

Table 4.1 Lipid recovery yield (% dry weight basis) of oil bodies recovered using different method [1]

Oil bodies [2]	% Lipid recovery [3]
MP-COB	14.6 ± 1.6 [b]
COB	30.0 ± 0.4 [a]
WWOB	12.7 ± 2.9 [bc]
UWOB	10.6 ± 2.2 [c]
Alkali-COB	28.9 ± 2.8 [a]
Alkali-WOB	2.8 ± 0.3 [d]

[1] Lipid recovery (%) = total lipid in oil bodies (g) / total lipid in rice bran (g) x 100

[2] MP-COB = mortar and pestle-crude oil bodies, COB = water-based crude oil bodies, WWOB = water-based water-washed oil bodies, UWOB = water-based urea-washed oil bodies, alkali-COB = alkali-based crude oil bodies, alkali-WOB = alkali-based washed oil bodies

[3] Values within columns followed by the different letter are significantly different ($p>0.05$, ANOVA) ($n = 3$, ± SD)

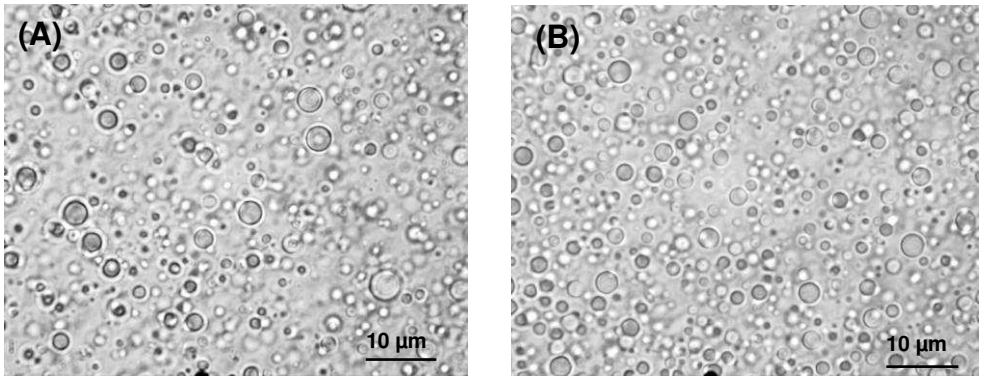


Figure 4.3 Light microscope images of rice bran oil body preparation using an alkali wash

A, alkali-crude oil bodies; B, alkali-washed oil bodies

The alkali-wash oil bodies were further observed under confocal microscopy (Figure 4.4). Fluorescence intensity of lipids in oil body suspension that were stained with Nile red, appeared in a green color (section 2.4.3). From the micrographs, alkali wash eliminates large or defective lipid droplets from crude oil body preparation and retains small uniform intact oil bodies.

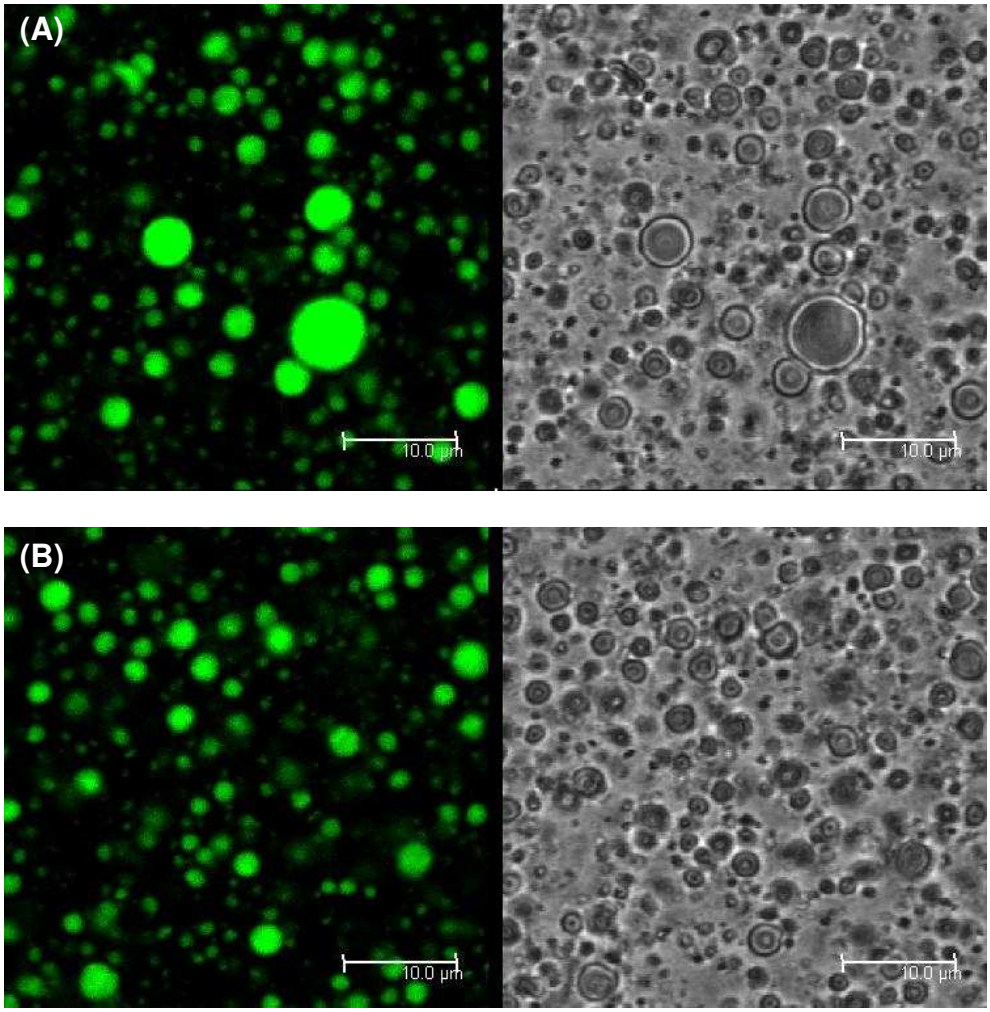


Figure 4.4 Confocal microscope images (left) of rice bran oil body preparation using an alkali wash compared with light microscope images (right) taken at the same location and magnification

A, alkali-crude oil bodies; B, alkali-washed oil bodies

4.1.4. Enzyme-assisted oil body recovery

Cell wall digesting enzymes were first tested for their ability to release oil bodies from rice bran. The enzymes selected for this trial included cellulase from *Trichoderma reseei*, cellulase from *Aspergillus niger* and papain from *Carica papaya*. An enzyme solution was made according to section 2.3.4. Rice bran (10 g) was mixed with 50 ml dialyzed enzyme solution (3 % w/w enzyme to rice bran) and incubated at 50 ºC, 150 rpm. After 2 h, the pH was adjusted to pH 8.0. The slurry was homogenized in a blender for 2 min. After centrifugation, the creamed oil body layer floating on top of the homogenate was carefully collected and labeled as enzyme-assisted crude oil bodies (enzyme-COB).

In order to compare the performance of each enzyme, total sugar released from the cell wall components into the reaction medium buffer was measured using the phenol-sulfuric acid method. The highest amount of total sugar released was found in the medium containing cellulase from *A. niger* (120 ± 7 mg glucose/g rice bran) (Table 4.2), but only a marginal difference was found between the total sugar released from cellulase (*A. niger*) and the total sugar contained in the blank solution, which did not contain any enzymes (93 ± 6 mg glucose/g rice bran). Furthermore, the amount of total sugar released was not related to the oil body lipid recovery.

Table 4.2 Chemical composition and lipid recovery yield (% dry weight basis) of enzyme-assisted oil body [1]

Enzyme	% Lipid [2]	% Lipid recovery [3]	% Protein	Total sugar [4] (mg Glc/g RB)
No enzyme [5]	54.1 ± 0.4 [c]	21.0 ± 3.2 [c]	31.9 ± 0.9 [a]	93 ± 6 [b]
Cellulase *T. reseei*	57.8 ± 0.6 [b]	29.1 ± 2.3 [b]	27.6 ± 0.4 [b]	102 ± 4 [b]
Cellulase *A. niger*	55.0 ± 0.2 [c]	21.0 ± 2.0 [c]	27.9 ± 0.2 [b]	120 ± 7 [a]
Papain	69.6 ± 0.8 [a]	52.1 ± 1.5 [a]	22.2 ± 0.8 [c]	96 ± 5 [b]

[1] Values within columns followed by the different letter are significantly different ($p>0.05$, ANOVA) ($n = 3$, ± SD)

[2] Lipid content (%) = lipid dry weight (g) / oil body dry weight (g) x 100

[3] Lipid recovery (%) = total lipid in oil bodies recovered after incubation (g) / total lipid in rice bran (g) x 100

[4] Total sugar release from rice bran in reaction medium (mg glucose/g rice bran)

[5] Oil bodies recovered by mixing rice bran in buffer without the use of enzyme as a control

Oil bodies recovered with the use of enzymes were low in lipid (54.1 - 69.6 %) and high in protein (22.2 - 31.9 %) as compared with oil bodies recovered by other methods in the previous sections. Papain yielded the highest lipid recovery (52.1 ± 1.5 %); the majority of the recovered lipid material was aggregated and coalesced as shown in a light micrograph (Figure 4.5D). There was also evidence of a significant amount of free oil present in this “oil body” fraction.

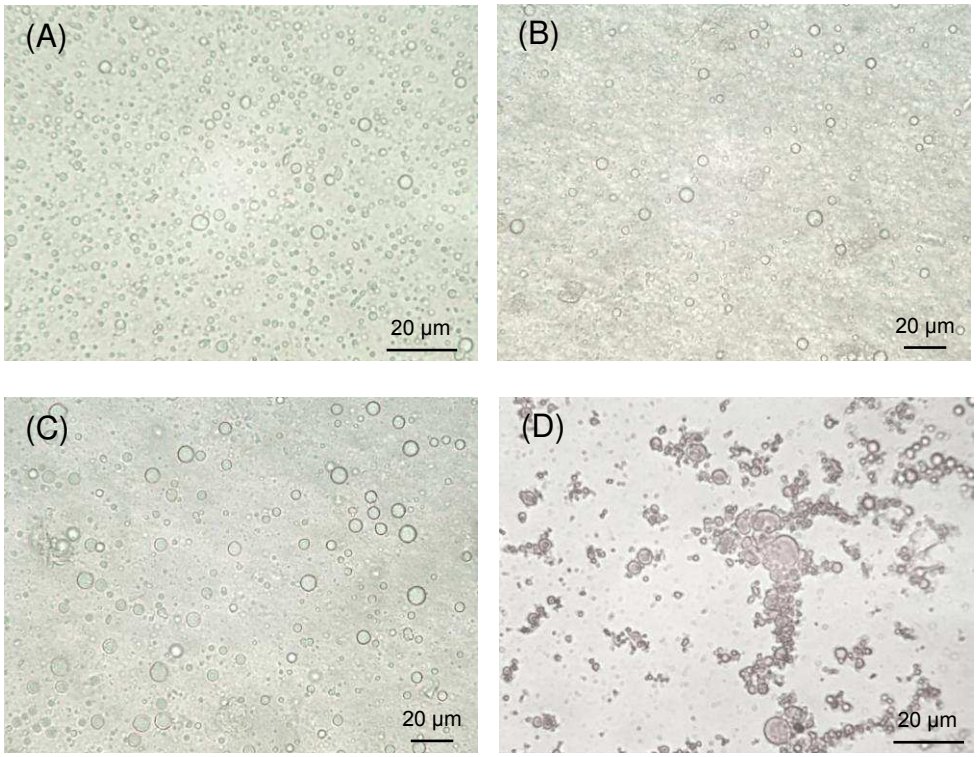


Figure 4.5 Light micrographs of enzyme-assisted oil body recovery. Oil bodies were recovered with the used of A), no enzyme; B) cellulase from *T. reseei*; C) cellulase from *A. niger*; and D) papain.

In the present study, rupturing of rice bran surface was created during wet milling. The rupturing of plant cell wall also occurs naturally during fruit ripening. Polygalacturonase (PGase, 100kDa) is activated in ripe fruits and can transfer through or within the plant cells (Buchanan *et al.*, 2000). Therefore, during wet milling, oil bodies may be released out or cell wall degrading enzymes may access into the break up cell. This promotes digestion of oil body surface protein (oleosin) by papain (a protease, 23 kDa), which increases the free oil and coalescence of oil bodies. In addition, other proteins in rice bran

such as albumin, globulin, glutelin and prolamin (Adebiyi *et al.*, 2009) could have been hydrolysed by papain. This also highlights the importance of oil body stabilizing proteins by steric hindrance.

The volume mean diameter of papain-COB droplets was extremely large (11.3 ± 0.1 μm), while those of cellulase-COB were smaller (5.2 - 7.9 μm). From the results of the lipid content, recovery yield, integrity of the oil bodies from micrographs and particle size analysis, it can be seen that the recovery of oil bodies with the treatment of cell wall degrading enzymes was not suitable for preparing oil bodies that were intact and enriched in lipids for our studies. Therefore, no further experiments were conducted from this method.

It is clear that rice bran oil bodies can be recovered by wet milling protocols. The mortar and pestle method is simple, but slow, tedious and uncontrollable. The enzyme-assisted method was not suitable because the yields of oil body lipid recovery were low and the recovered oil bodies were less intact and damaged. The potential methods that could recover intact oil bodies are the water-based (see section 4.1.2. for details) and alkali-based methods (section 4.1.3.). The compositional and physical characteristics of crude and purified oil bodies recovered from these two methods are compared and discussed in the following sections.

4.2. Chemical composition

4.2.1. Basic composition

Oil bodies were recovered from rice bran by wet milling with either water (water-based method) or alkali solution (alkali-based method), filtration and centrifugation to produce buoyant crude oil body (COB) material floating on top of the homogenate. The crude oil bodies were further purified by washing with various media to remove cell debris, extraneous proteins and other contaminants. In the water-based method, crude oil bodies were washed with water to produce water-washed oil bodies (WWOB) or 9 M urea to produce urea-washed oil bodies (UWOB). In the alkali-based method, crude oil bodies (alkali-COB) were washed with 0.1 M $NaHCO_3$ to produce alkali-washed oil bodies (alkali-WOB). The lipid recovery yields (% dry weight basis) are shown in Table 4.1. The basic compositions of crude and washed oil bodies are compared in Table 4.3.

Table 4.3 Lipid and protein content (% dry weight basis) of crude and washed oil bodies recovered from water-based and alkali-based method

Oil bodies	Lipid content (%)	Protein content (%)
COB	81.0 ± 1.1	12.7 ± 0.3
WWOB	87.0 ± 1.8	6.5 ± 0.6
UWOB	93.0 ± 2.2	3.6 ± 0.6
Alkali-COB	83.7 ± 3.1	11.5 ± 0.7
Alkali-WOB	96.8 ± 2.6	1.2 ± 0.1

Oil bodies washed with 9 M urea were significantly ($P<0.05$) enriched in lipid (93.0 ± 2.2 % dry wt.) and low in protein (3.6 ± 0.6 %) compared with unwashed (81.0 ± 1.1% lipid; 12.7 ± 0.3% protein) and water washed (87.0 ± 1.8% lipid; 6.5 ± 0.6% protein) oil body material. The results indicated that 9 M urea is an effective chaotropic agent to remove extraneous protein from rice bran oil bodies. Protein content of oil bodies recovered from the alkali-method was further reduced after washing with another alkali solution (0.1 M $NaHCO_3$) (96.8 ± 2.6 % lipid and 1.2 ± 0.1 % protein). From the results, the alkaline solution (0.1 M $NaHCO_3$) was the most aggressive washing media as compared with water and 9 M urea as it removed more proteins. It is clear that the effective lipid concentration increased, while protein concentration decreased after washing oil bodies from the rice bran; similar findings have been reported for oat oil bodies (White *et al.*, 2006).

4.2.2. Protein composition

Gel electrophoresis of the purified protein fraction of oil bodies recovered using either the water-based and alkali-based method revealed similar protein profiles (Figure 4.6 and Figure 4.7). However, there is a relative increase in the band density of washed oil bodies (labeled as Lane 3 and 4, Figure 4.6) compared with that of crude oil bodies (labeled as Lane 2, Figure 4.6). These were corresponding to the protein with the mass of 16 kDa (band J) and 18 kDa (band I),

which were the candidates of oleosin. Several protein bands were present in these preparations suggesting that washed oil body preparations were not entirely free of contaminating protein. In addition, the small protein bands (10 - 12 kDa, bands K – L) were not found in the alkali-WOB (Figure 4.7) compared with the WWOB and UWOB (Figure 4.6), indicating that washing protocol of the alkali-based method removed more extraneous protein from oil bodies than that of the water-based method. The fact that the bands from the purified oil body material are consistent with the known composition of oil bodies (Chen *et al.*, 1998; Huang, 1992) suggests that the oil bodies have been purified correctly.

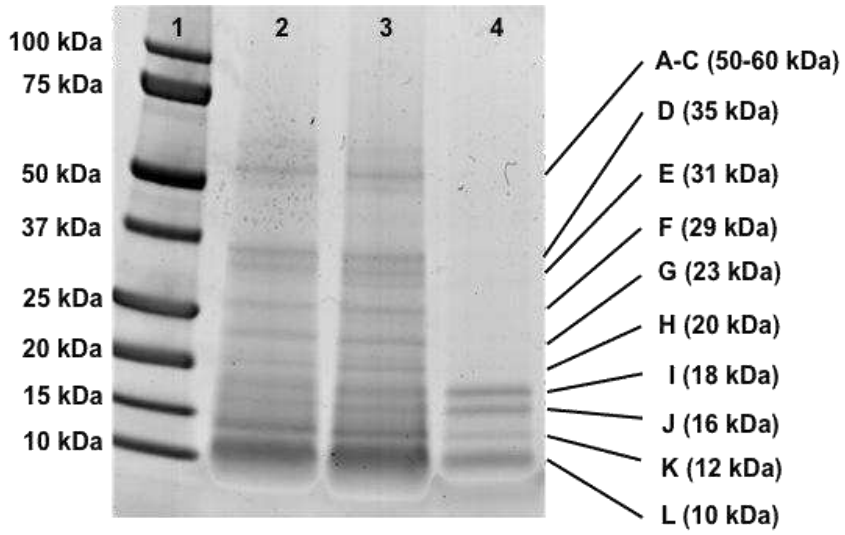


Figure 4.6 SDS-PAGE profiles in oil body preparations from rice bran by water-based method.

Lane 1, molecular weight marker; lane 2, crude oil bodies; lane 3, water-washed oil bodies; and lane 4, urea-washed oil bodies. The tentative identification of bands by molecular weights: A - H are unknown, I - J are oleosin isoforms, K and L are unknown.

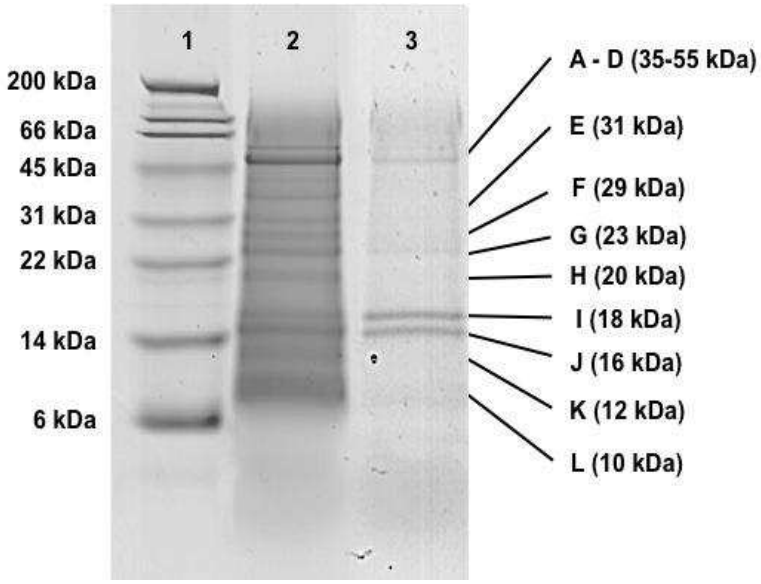


Figure 4.7 SDS-PAGE profiles in oil body preparations from rice bran by alkali-based method.

Lane 1, molecular weight marker; lane 2, alkali-crude oil bodies; and lane 3, alkali-washed oil bodies. The tentative identification of bands by molecular weights: A - H are unknown, I - J are oleosin isoforms, K and L are unknown.

Whether or not the unknown bands provide evidence for aggregations (i.e. dimers, trimmers) of known proteins, unknown oil body proteins or fragments of known proteins respectively is not clear. Adebiyi *et al.* (2009) reported the molecular weights of rice bran albumin, globulin, glutelin and prolamin were in the range of 30 - 45, 20 - 66, 10 - 66, and 10 - 53 kDa respectively. The ratio of proteins in rice bran has been reported to be albumin 37%, globulin 36%, glutelin 22% and prolamin 5% (Luh *et al.*, 1991). The protein bands of higher molecular weight than oleosins (20 - 55 kDa) could be albumin and globulin since they are abundant in rice bran and are

extracted under the conditions of oil body recovery in this study. Since glutelin is alkaline-soluble protein, it can be extracted with the oil bodies and is possibly absorbed on the oil body surface, but was removed by the alkali-washing solution. Prolamin may not be extracted under the condition of oil body recovery because it is very low in rice bran and soluble in alcohol. In addition, the major soluble basic protein including cytochrome C (12 kDa) and a blue protein (a copper-containing glycoprotein, 18.3 kDa) were also isolated from bran (Ida and Morita, 1969). However, these proteins can be removed by chelating agent (EDTA) in the alkaline washing protocol. Therefore, albumin and globulin may be the tentative protein fractions of the unknown bands. These proteins may be covered or absorbed on the oil body surface and may be attributed to the characteristic of the oil body suspension.

Because of the presence of surface proteins and the association of these proteins and the phospholipid monolayer, oil bodies are intrinsically stable *in vivo* and allows the recovered crude oil bodies to remain as single entities *ex vivo* (Figure 4.2A). However, washing oil bodies with water or 9 M urea resulted in much larger droplets and coalescence of oil bodies (Figure 4.2C). In addition, the formation of oil layer on the top of urea washed materials or “oiling off” was observed indicating that coalescence had occurred. The 9 M urea wash was strong enough to remove some of the oleosin or

other extraneous proteins from the oil body surface suggesting that the extraneous proteins along with oleosin attributed to the stability of rice bran oil bodies against coalescence. It is possible that oil bodies in cereal, which in this case is rice bran, are more fragile than oil bodies from their oil seed cousins such as sunflower seeds. It has been reported that urea washing has no effects on the stability of oil bodies recovered from sunflower seed (White *et al.*, 2008) and conformation of oleosins in intact oil bodies from oilseeds (safflower and sunflower) (Lacey *et al.*, 1998).

The oil body purification by alkali washing protocol can remove the defected oil bodies, leaving behind intact oil bodies as can be seen by the very low lipid recovery yield (2.8 ± 0.3 %, Table 4.1), the small size (Figure 4.3), and the remaining of oleosin on alkali-WOB as seen on SDS-PAGE (Figure 4.7).

Urea is widely known to have a denaturing effect on proteins at high concentrations by disrupting non-covalent bonds of the proteins (Stryer, 2000). Sodium bicarbonate induces denaturation by unfolding extraneous proteins from surface of oil bodies leading to a decrease in protein concentration. In addition, the alkaline solution may react with other acidic impurities and remove them from the crude preparation.

4.2.3. Lipid classes

The major lipid in oil bodies (Figure 4.8, lane 1 - 3) was triacylglycerols, while that of rice bran (lane 4) was free fatty acids. The high amount of free fatty acids in freshly milled rice bran in this study may be explained by the age of the brown rice (more than 1 year old) from which the bran was derived. Free fatty acids were removed successively during oil body recovery as can be seen from the increased density of free fatty acid bands in the coarse residue, fine residue and supernatant lipid fractions (lane 5 - 7). After alkali washing, the concentration of free fatty acids in washed oil bodies was reduced (lane 3). This was supported by the results from free fatty acid determination by spectrophotometry (Figure 4.9). It shows that alkali washing is an effective method to remove free fatty acids from the oil body preparation.

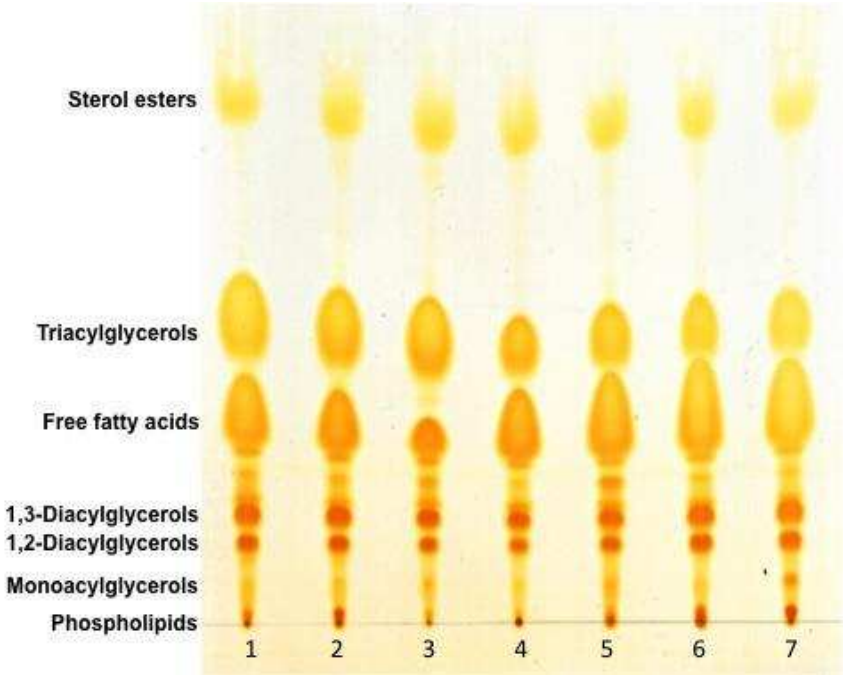


Figure 4.8 Separation of rice bran lipid classes on TLC plate (Silica Gel 60)

Developing solvent: hexane-diethyl ether-acetic acid (80:20:2 by volume). 1, COB lipids; 2, alkali-COB lipids; 3, alkali-WOB lipids; 4, rice bran lipids; 5, coarse rice bran residue lipids; 6, fine rice bran residue lipids; 7, supernatant lipids

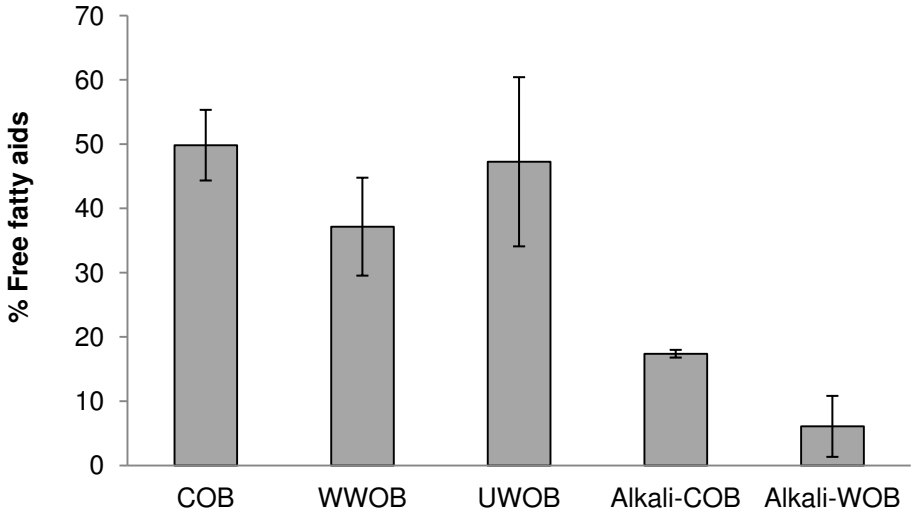


Figure 4.9 Free fatty acid content (% oleic acid) in lipids of crude and washed oil bodies recovered from water-based and alkali-based method

4.2.4. Fatty acid composition

Like the protein composition data (section 4.2.2.), the fatty acid composition of washed oil bodies is similar to that of the crude oil bodies and thus washing the crude oil body material has no effect on it (Figure 4.10 and 4.11). Oleic acid is the major fatty acid found in rice bran oil bodies, followed by linoleic acid, palmitic acid, stearic acid and linolenic acid respectively. Approximately 75% of fatty acids present are unsaturated. In addition, the fatty acid composition of oil bodies is very similar to that of parental rice bran.

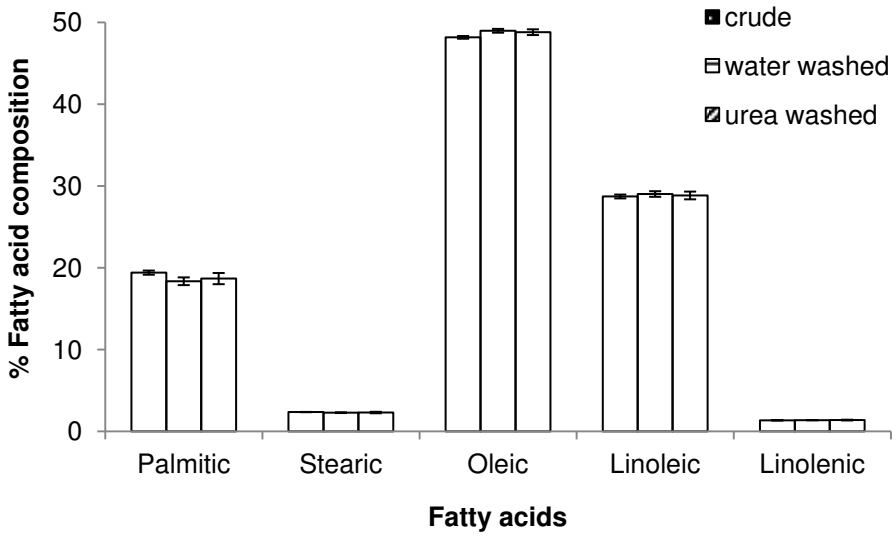


Figure 4.10 Fatty acid composition in crude oil bodies and oil bodies after washing with water and 9 M urea

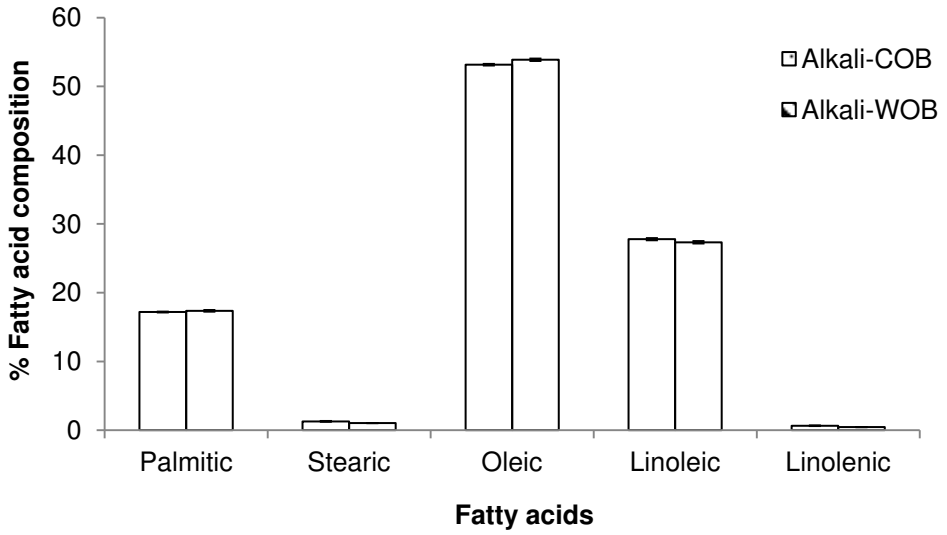


Figure 4.11 Fatty acid composition in alkali-crude oil bodies (alkali-COB) and oil bodies after washing with 0.1 M $NaHCO_3$ (alkali-WOB)

4.2.5. Phytochemical composition

Analysis of tocochromanols and oryzanol by RP-HPLC showed that these lipophilic phytonutrients were concentrated in rice bran and corresponding oil bodies (Table 4.4) suggesting that recovered oil bodies are enriched in phytonutrients. The association between tocochromanols and oryzanol in rice and rice bran oil bodies was further studied by washing the oil bodies to remove weakly-associated compounds. An indication of the integral association between the phytochemicals and the oil bodies can be made by comparing the concentration of these compounds per unit mass of lipid in the starting material with those in the purified oil bodies. Based on the unit mass of lipid in crude oil bodies, high

concentrations of tocochromanols (76 - 100 %) and oryzanol (91 - 98 %) were retained after washing with water and urea as compared with crude, unwashed oil bodies in the water-based method (Table 4.5). Using the alkali-based method, high concentrations of tocochromanols (97 %) and oryzanol (96 %) were retained after washing with the alkaline solution. These results strongly suggests that the majority of oil rice bran tocochromanols and oryzanol are strongly associated with oil bodies, as the hydrophobic side chain of these molecules would be inserted into the lipid core of the oil body and the polar head group exposed to the surface.

Table 4.4 Phytochemical composition in rice bran, crude oil bodies and washed oil bodies

Phytochemical [1]	Concentration (mg/kg lipid) [2]					
	Rice bran	COB	WWOB	UWOB	Alkali-COB	Alkali-WOB
δ-T3	153 ± 5	90 ± 2	89 ± 2	82 ± 2	76 ± 5	79 ± 6
β/γ-T3	771 ± 18	425 ± 9	429 ± 7	316 ± 7	688 ± 6	721 ± 6
α-T3	116 ± 10	48 ± 5	45 ± 4	33 ± 1	69 ± 1	52 ± 2
δ-T	9.4 ± 0.6	4.8 ± 0.2	5.6 ± 0.1	4.3 ± 1.4	9.8 ± 0.3	8.6 ± 3.3
β/γ-T	104 ± 5	30 ± 1	30 ± 1	22 ± 1	66 ± 1	62 ± 3
α-T	277 ± 15	58 ± 4	61 ± 1	41 ± 1	96 ± 2	52 ± 16
Total T3	1,040 ± 22	563 ± 4	562 ± 1	432 ± 2	833 ± 8	852 ± 9
Total T	390 ± 16	93 ± 10	96 ± 8	68 ± 7	172 ± 2	122 ± 17
Total	1,430 ± 27	656 ± 11	658 ± 8	499 ± 7	1,006 ± 8	974 ± 19
Oryzanol	14,174 ± 245	9,692 ± 130	9,538 ± 44	8,830 ± 97	8,880 ± 35	8,503 ± 48

[1] T3 = tocotrienols; T = tocopherol; Total = sum of tocotrienols and tocopherols
[2] The values shown are mean ± SD of three replicates, calculated on a dry weight basis

In contrast, there was a significant decrease ($P<0.05$) in the total phenolic content (TPC) in the washed oil bodies compared to the crude oil bodies (Figure 4.12). This demonstrates that there is a significant pool of phenolic compounds that are removed during washing steps along with extraneous proteins except that of alkali-WOB. The phenolic compounds were recovered with the alkaline solution and remained with the alkali-WOB at high concentration.

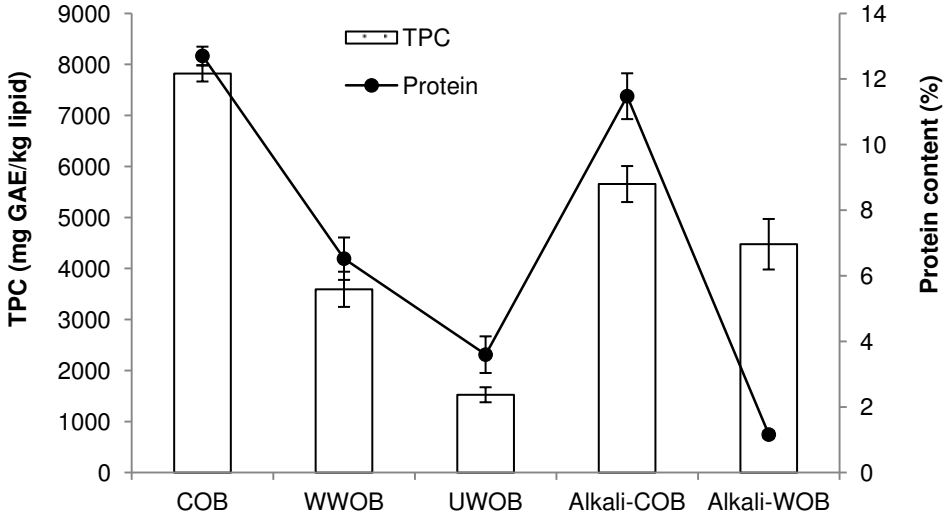


Figure 4.12 Total phenolic content (TPC) and protein content (% dry weight basis) of crude and washed oil bodies by water-based and alkali-based method

Furthermore, the reduction in total phenolic content is relatively large with respect to the reduction in tocochromanols and oryzanol (Table 4.5). In the water-based method, 80 % of TPC was lost from UWOB, while only 24% of tocochromanols and 9 % of oryzanol were lost from UWOB. Therefore, tocochromanols and

oryzanol appear to be physically associated with rice bran oil bodies *ex vivo* and presumably *in vitro* as well. These results agree with a similar study of *Avena sativa* that posited an intrinsic association between tocochromanols and oil bodies in the seeds in that species (White *et al.*, 2006).

Table 4.5 Retention of phytochemical concentration between crude and washed oil bodies [1]

Phytochemical [2]	Phytochemical retention between crude and washed oil bodies (%)				
	COB	WWOB	UWOB	Alkali-COB	Alkali-WOB
Tocochromanols	100	100 ± 2	76 ± 2	100	97 ± 2
Oryzanol	100	98 ± 1	91 ± 2	100	96 ± 1
TPC	100	46 ± 5	20 ± 2	100	79 ± 10

[1] Retention (%) = Phytochemical concentration in washed oil bodies (mg/kg lipid) divided by phytochemical concentration in crude oil bodies (mg/kg lipid) x 100
[2] Tocochromanols = sum of tocotrienols and tocopherols; TPC = total phenolic content

4.3. Imaging and size of oil bodies

4.3.1. Imaging of rice bran and oil bodies

Transmission Electron Microscopy (TEM, see section 2.4.2) was used to observe rice bran oil bodies *in vivo*. Thin sections (0.5 µm) of brown rice were cut and stained with toluidine blue for light microscopy (Figure 4.13) before the ultra-thin sections (80 nm) were selected for TEM (Figure 4.14A). Oil bodies appear as light grey spherical droplets in brown rice and all were 0.5 - 1 µm in diameter.

White large particles of 5 - 10 µm are starch granules and other small particles in purple are protein bodies. The oil bodies were compressed within cells but remained as individual organelles. In general, oil bodies were observed in the aleurone and sub-aleurone layers, which are commonly found in the bran layer (Figure 4.14B). The distribution of oil bodies observed is in agreement with previous studies on oil bodies from rice (Wu *et al.*, 1998) and oat grain (White *et al.*, 2006).

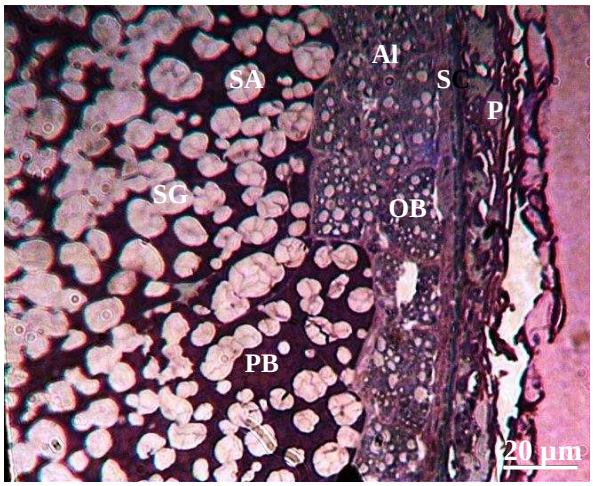
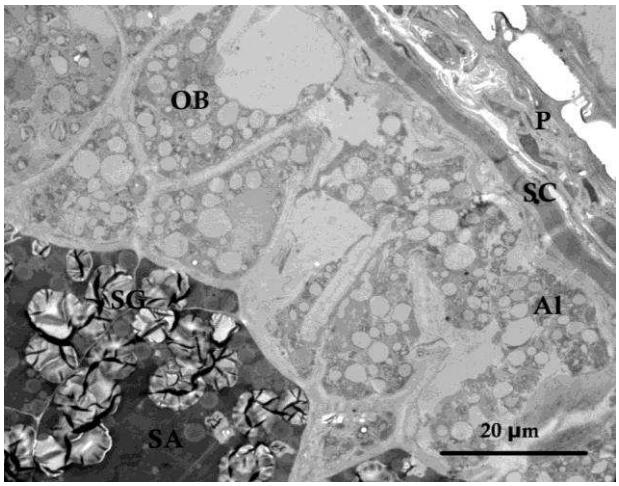


Figure 4.13 Light micrograph of brown rice thin section (0.5 µm) stained with toluidine blue

OB, oil bodies; P, pericarp; SC, seed coat; Al, aleurone; SA, sub-aleurone; SG, starch granule; PB, protein bodies

(A)



(B)

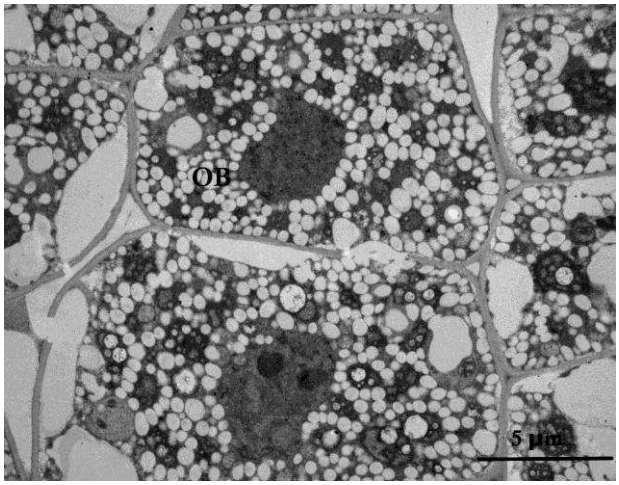


Figure 4.14 Transmission electron micrographs of oil bodies in (A) brown rice surface and (B) aleurone region of brown rice

OB, Oil bodies; P, Pericarp; SC, Seed coat; Al, Aleurone; SA, Sub-aleurone; SG, Starch granule

4.3.2. Particle size

Volume mean diameters D(4,3) (see section 2.9.2) of crude, water-washed and urea-washed oil bodies were 4.4 ± 0.0, 9.9 ± 0.1 and 12.7 ± 0.6 µm respectively, which may have led to an increase in droplet aggregation and coalescence after washing with 9 M urea. Alkali-COB had a volume mean diameter of 4.5 ± 0.2 µm, and once washed, this was reduced to 1.9 ± 0.0 µm. The volume distributions of oil body suspension from the water-based and the alkali-based method are shown in Figure 4.15 and 4.16 respectively.

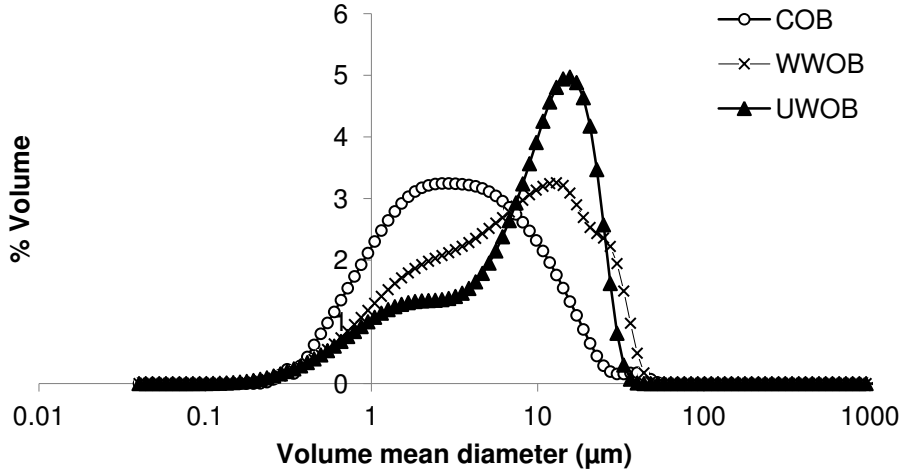


Figure 4.15 Particle size distribution of rice bran oil body

COB, crude oil bodies; WWOB, water-washed oil bodies, UWOB, urea-washed oil bodies

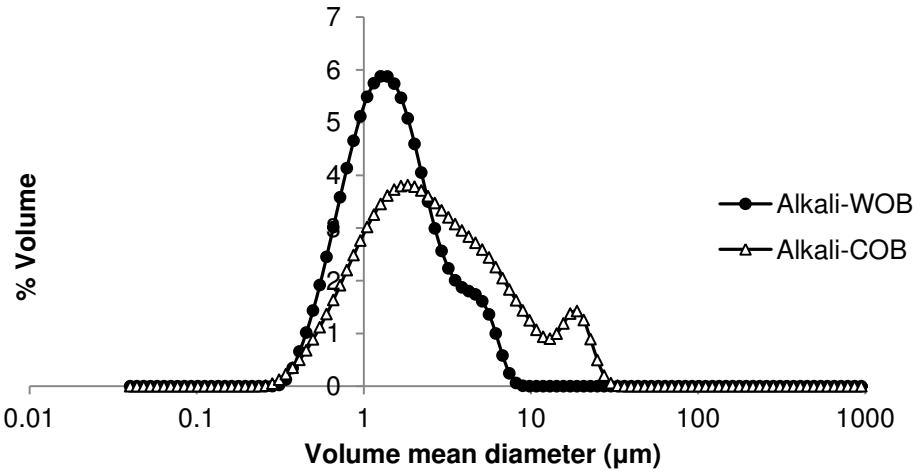


Figure 4.16 Particle size distribution of rice bran oil body preparation using an alkali wash

Alkali-COB, alkali-crude oil bodies; Alkali-WOB, alkali-washed oil bodies

In addition, some previous work on emulsion measured the droplet size based on surface mean diameters D(3,2) (Iwanaga *et al.*, 2007) and median values from a volume distribution Dv50 (Beisson *et al.*, 2001). Table 4.6 shows the oil body particle size by various approaches in order to describe the distribution of the samples.

Table 4.6 Particle size of oil bodies by various approaches

	D(4,3) [1]	D(3,2) [2]	Dv50 [3]
COB	4.4 ± 0.0	2.0 ± 0.0	2.8 ± 0.0
WWOB	9.9 ± 0.1	3.1 ± 0.0	5.2 ± 0.0
UWOB	12.7 ± 0.6	4.2 ± 0.1	11.0 ± 0.7
Alkali COB	4.6 ± 0.2	1.8 ± 0.0	2.5 ± 0.1
Alkali WOB	1.9 ± 0.0	1.3 ± 0.0	1.5 ± 0.0

[1] D(4,3) = volume mean diameter
[2] D(3,2) = surface mean diameter
[3] Dv50 = median based on volume distribution

However, it appears that the particles size of the isolated oil bodies measured from a particle sizer (diameter of 4 - 5 μm) were larger than they appeared *in vivo* (0.5 - 1 μm, Figure 4.14). The volume mean diameter can over estimate the mean diameter due to the weighting on volume. Despite this limitation, it is clear that washing oil bodies from rice bran results in an increase in droplet size. It is possible that rice bran oil bodies are fragile *ex vivo* and therefore, coalesce *ex vivo*.

4.4. Stability of oil body suspension

4.4.1. pH stability

The understanding of physical stability is very important when the oil bodies are applied commercially. Their physical stability will also determine their structural integrity during processing, storage, transportation and utilization. A series of experiments were carried out to determine the influence of pH on the stability of oil bodies. The stability of oil body suspension from rice bran was analyzed using light micrograph, particle size, relative turbidity and zeta-potential measurements (see section 2.9). Oil bodies once recovered can be re-dispersed in media to make a suspension. In order to study the electrostatic stabilization of rice bran oil bodies, crude oil bodies recovered from both water-based (diameter of 4.4 ± 0.0 μm) and alkali-based (diameter of 4.5 ± 0.2 μm) methods were dispersed in phosphate-citrate buffers using

Potter-Elvehjem homogenizer over a range of pH values (2 - 8). The final pH of the oil body suspensions was checked and adjusted to the desired pH, if necessary. Figure 4.17 shows micrographs of the oil bodies dispersed in buffer at different pH values.

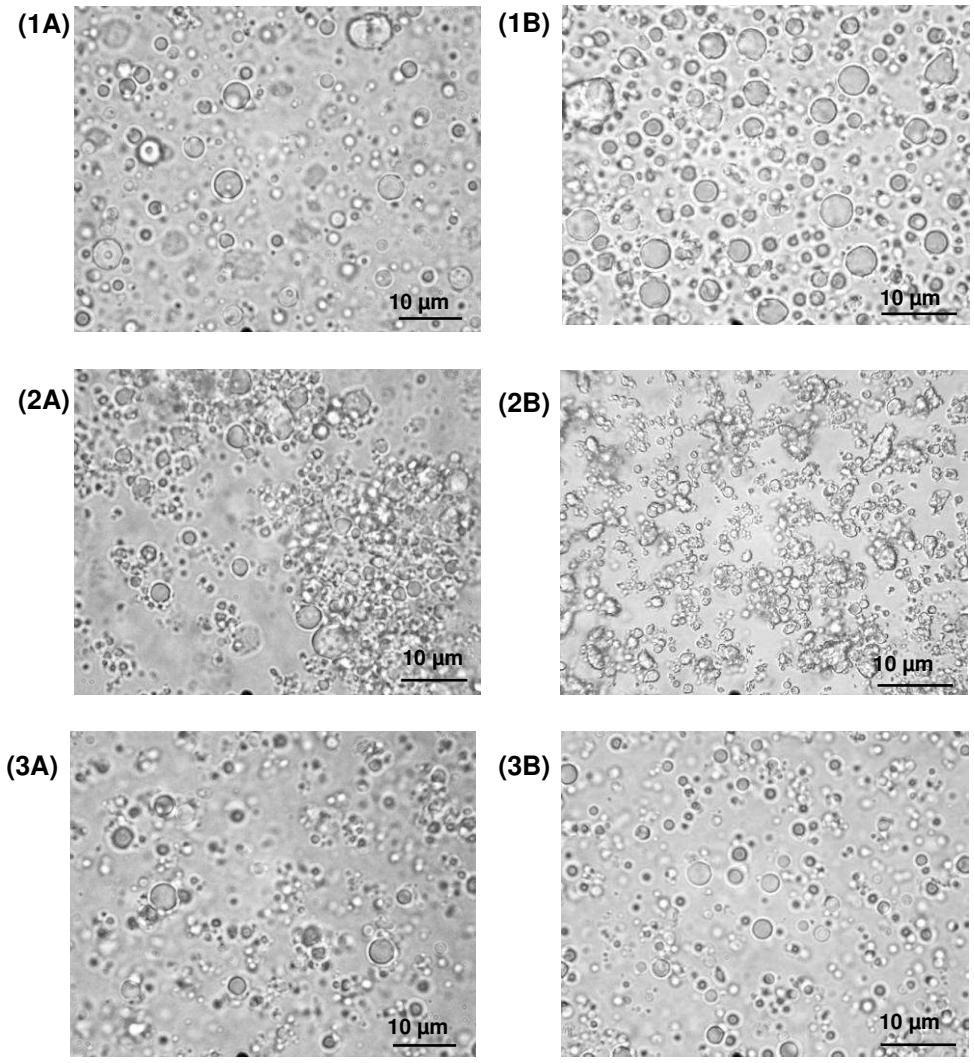


Figure 4.17 Light micrographs of (A) crude oil bodies and (B) alkali-crude oil bodies at selected pH values

1, pH 2; 2, pH 4 and 3, pH 8

The volume mean diameters of crude oil body suspensions at selected pH values are presented in Figure 4.18 and Figure 4.19. The oil body suspension at pH 4 is unstable and extremely large (more than 10 μm) because of the aggregation and coalescence of the oil body droplets (Figure 4.17 2A and 2B).

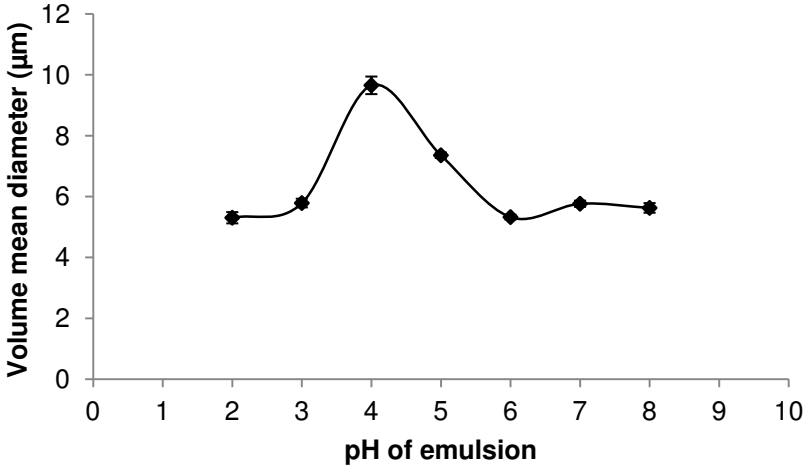


Figure 4.18 Particle size of crude oil bodies at selected pH values

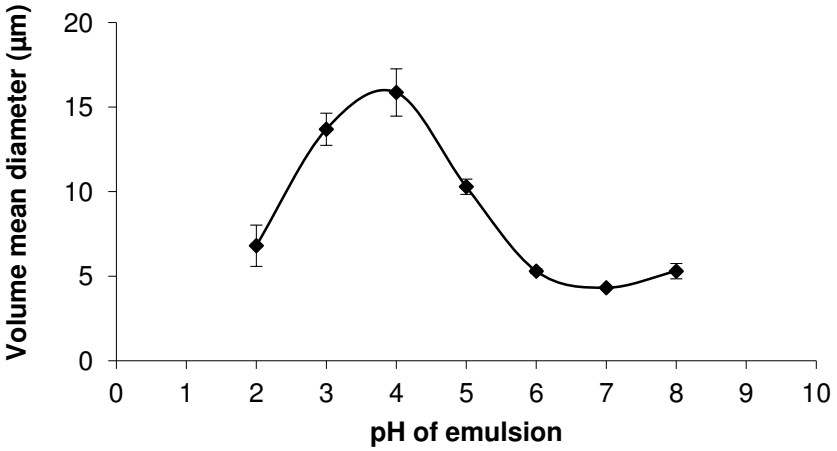


Figure 4.19 Particle size of alkali-crude oil bodies at selected pH values

4.4.2. Turbidity test

Oil body suspension is known to be thermodynamically unstable which leads to rapid phase separation (McClements, 1999). A layer of cream (lower density oil bodies / oil droplets) floats on top layer of water (higher density). As a result, turbidity of the suspension decreases. Despite the oil bodies slowly creaming to the top of the dispersion, the crude and washed oil body suspensions were quite stable and their relative turbidity only changed negligibly over 6 hours at room temperature (Figure 4.20). In addition, the pH of each oil body suspension did not change during the investigated period.

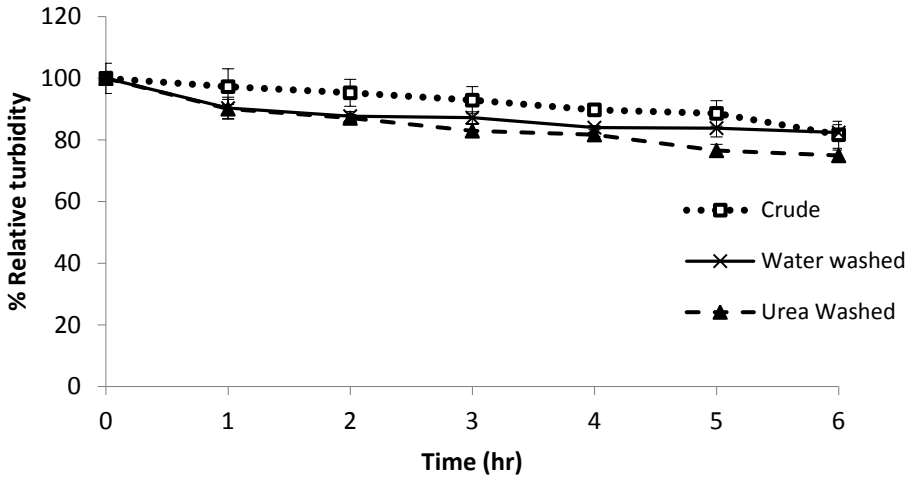


Figure 4.20 Relative turbidity of suspension of oil bodies recovered by water-based method over time

Crude oil body suspension in distilled water (pH 6.7),
Water-washed oil body suspension in distilled water (pH 6.8), and
Urea-washed oil body suspension in distilled water (pH 7.1)

The point of lowest relative turbidity is proposed to coincide with the isoelectric point (pI) of the oil body suspension. The isoelectric point is the pH where the positive and negative charges on the surface of the droplets balance and has no net electrical charge. At this point, there is no electrostatic repulsion between neighboring droplets to prevent oil body aggregation and cream separation. Figure 4.21 shows the relative turbidity of oil body suspension over 6 hours. For the crude oil bodies, the least relative turbidity and maximum aggregation were found at pH 4. The clear separation between cream phase and serum phase was observed in suspension at pH 4 - pH 5 (Figure 4.22). Similar results of maximum aggregation at pH 4 were found from alkali-crude oil bodies (data not shown).

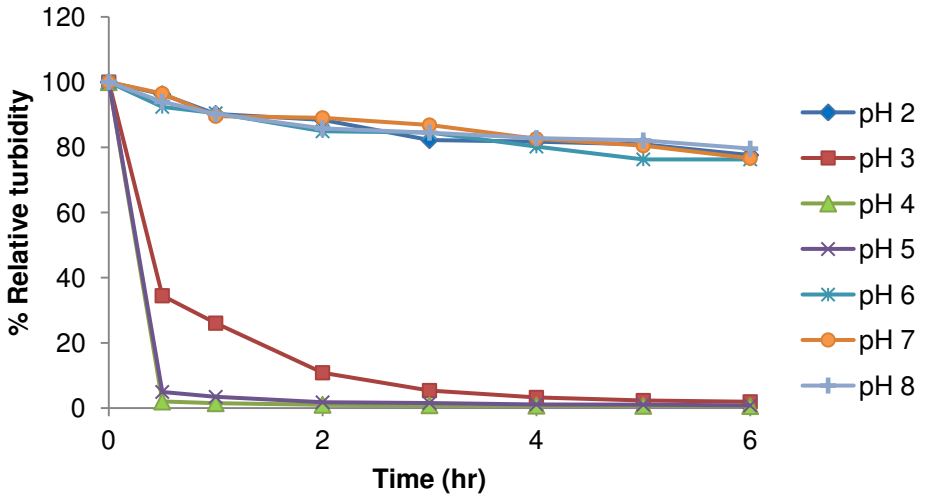


Figure 4.21 Relative turbidity of crude oil body suspension at different pH during 6 hours

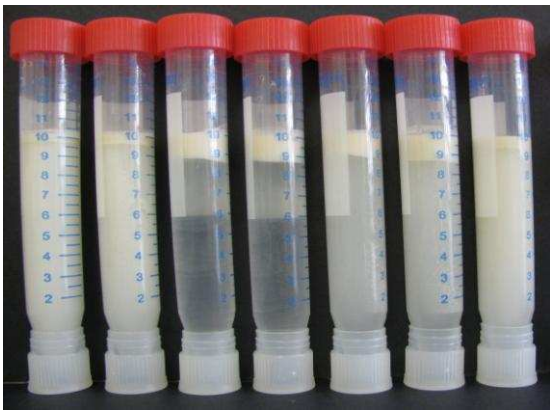


Figure 4.22 Stability test of oil body suspension at different pH after 6 hours at room temperature

4.4.3. Zeta-potential

Zeta-potential is widely used for determination of the magnitude of the electrical charges at the interfacial double layer (shear plane). In other words, zeta potential is the potential difference between the stationary layer of fluid (stern plane) attached to the dispersed particles and dispersed fluid in the diffuse layer (Figure 4.23).

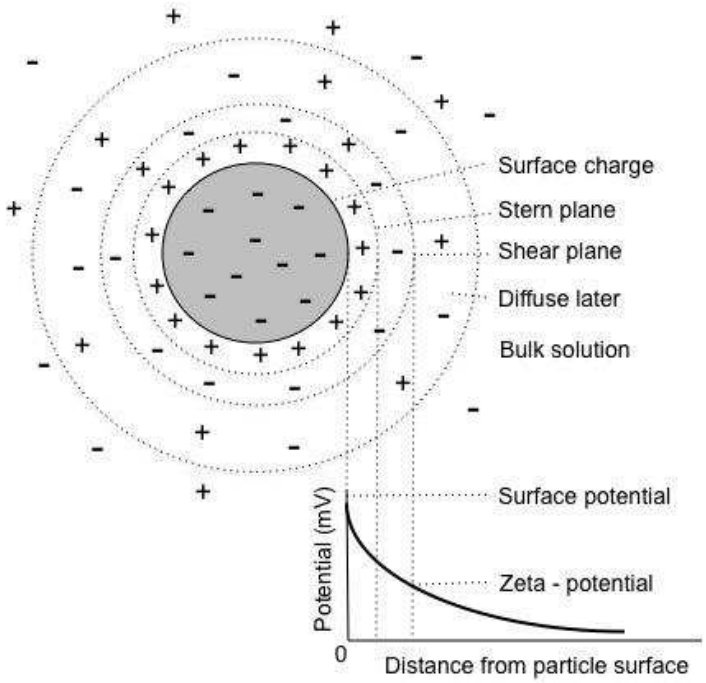


Figure 4.23 Zeta potential-potential difference as a function of distance from particle surface (adapted from BeckmanCoulter, Inc., 2010)

As velocity of charged particles in suspension is proportional to the amount of charge of the particles, zeta potential is determined by measuring the velocity of charged particles that moved toward an electrode opposite to their surface charges under an applied electric field. In order to determine the speed of particles in an electrophoretic light scattering method, particles in a flow cell are first irradiated with laser light and then the scattered light emitted from particles is detected. The mobility of particles can be measured from the frequency of the scattered light that shifted from the incident (laser) light, then the zeta potential can be calculated from this data (Figure 4.24).

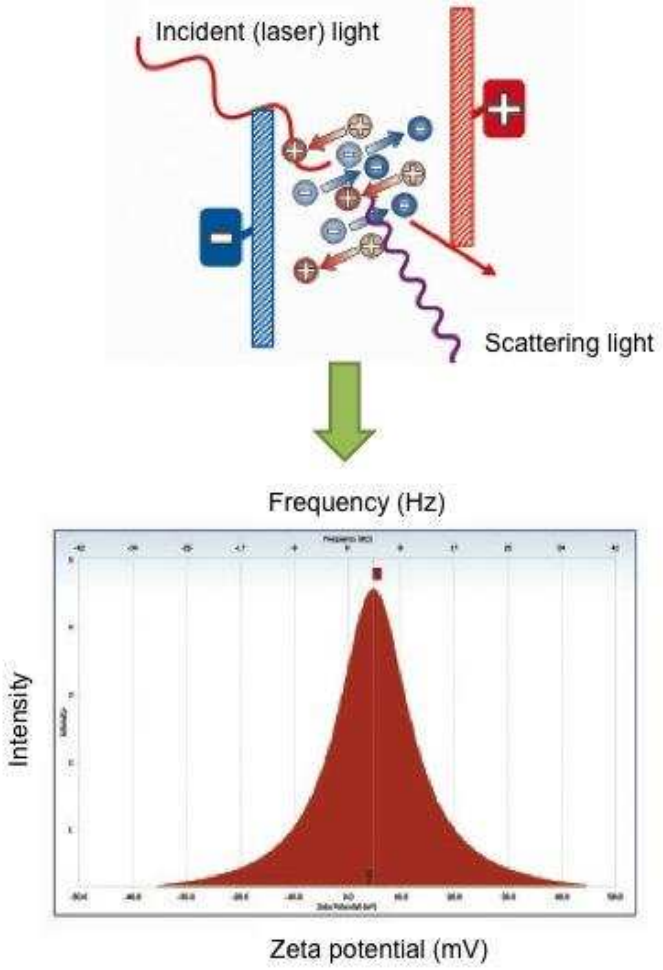


Figure 4.24 Determination of velocity of particles from the shift of frequency of the scattered light in electrophoretic light scattering method (adapted from BeckmanCoulter, Inc., 2010)

Zeta potentials of different oil bodies suspended in distilled water are compared in Figure 4.25. All of the prepared oil body suspensions had a negative surface charge. It was proposed that the electrostatic repulsion on the oil body surface was close to the minimum force for keeping oil bodies separate from each other and maintained them as discrete organelles in suspension (Chuang *et al.*, 1996). The zeta-potentials of crude, water washed and urea washed

oil body suspension are similar (about -30 ± 2 mV) indicating that the removal of extraneous proteins using water and urea was not related to the change in surface charge of the washed oil bodies.

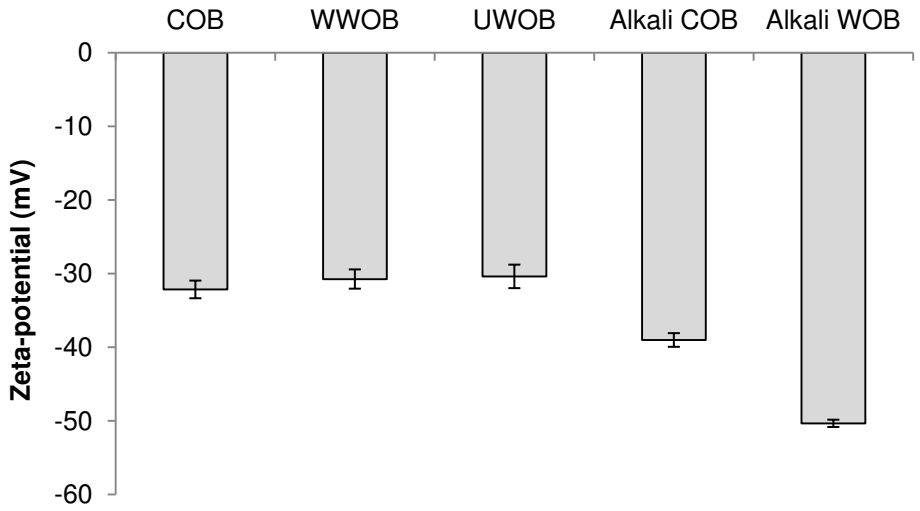


Figure 4.25 Zeta-potential of various oil bodies suspended in distilled water

COB, crude oil bodies (pH 6.34);
WWOB, water-washed oil bodies (pH 6.42);
UWOB, urea-washed oil bodies (pH 6.70);
Alkali-COB, Alkali-crude oil bodies (pH 6.99);
Alkali-WOB, Alkali-washed oil bodies (pH 6.32)

However, the zeta potential of the alkali-WOB increased significantly after washing (-50 mV). The increase in the magnitude of the electrical charge on the oil bodies after alkali washing suggested that the composition and/or the structure of surface layer around the oil bodies were changed. It is likely due to the removal of free metal ions such as Na^{+} and Ca^{2+} by EDATA and the removal of alkaline

soluble proteins, including glutelin, cytochrome C and blue protein, from the crude oil body by alkali washing solution (as seen on SDS-PAGE, Figure 4.7). The absent of positively charged metal ions and the basic proteins contributes to the loss of positive charges from the alkali-WOB surface. The remaining negatively charged phospholipids (phosphatidylserine and phosphatidylinositol) and free fatty acids (all having a pK greatly below 6.0) on the washed oil body surface, therefore, influence the overall surface charge of the washed oil body droplets and provide more negative charge.

The negatively charged surface of oil bodies has been reported previously (Tzen *et al.*, 1992). At neutral pH, positively charged amino acids (arginine, histidine and lysine) composed in oleosin are proposed to be located near the interior of the oil body surface where negatively charged phospholipids and free fatty acids are presented. The exterior of oleosin is mainly composed of negatively charged amino acids (aspartic acid and glutamic acid) giving the oil body an overall negatively charged surface.

As the pH of the media becomes close to the isoelectric point (pI) of the oil body suspension, the zeta-potential reduces to zero and the oil bodies do not move in the suspension under the applied electric field. From Figure 4.26, the point where zeta-potential of crude oil bodies approaches zero is between pH 4 - 5, while for

alkali-crude oil bodies is around pH 4. From the result of relative turbidity and zeta-potential measurement, it can be concluded that isoelectric point of rice bran crude oil body suspension is at pH 4 - 5 depending on the recovery and washing protocol. At this point, oil body suspension has the least physical stability; aggregation and creaming can occur rapidly. The aggregation at pH 4 - 5 can be related to the fact that zeta-potential of the oil bodies was relatively low and therefore, the electrostatic repulsion between droplets was not sufficient to prevent aggregation and creaming.

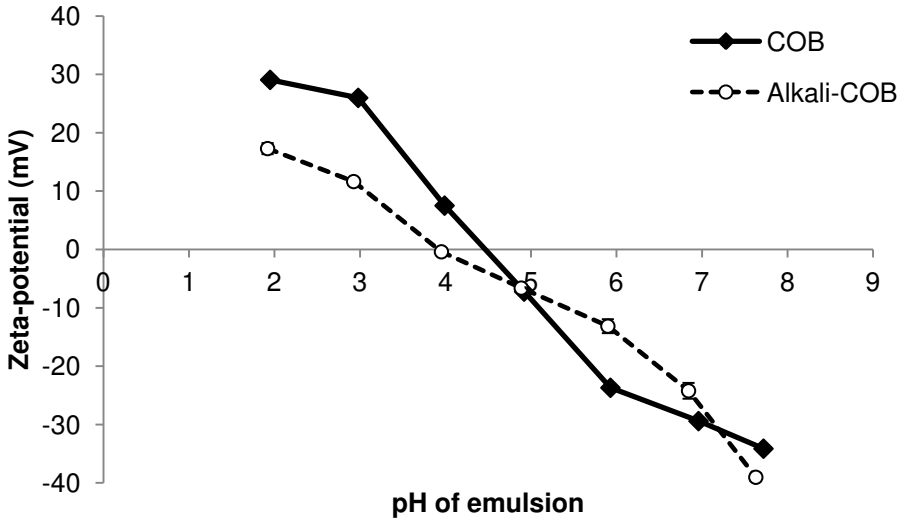


Figure 4.26 Zeta-potential of rice bran oil bodies

COB, crude oil bodies; Alkali-COB, alkali-crude oil bodies

Previous studies have found that the isoelectric point of oil bodies from various species was observed visually around pH 6 under isoelectric focusing (Chuang *et al.*, 1996; Tzen *et al.*, 1992), which is about 1 to 2 unit higher than the zero charge point of rice

bran oil bodies in this study (pH 4 - 5). The lower pI in this study may be attributed to the different method used, the absence of some basic extraneous protein and the presence of free fatty acids around the surface of rice bran oil bodies. The lower pI of oil bodies at around pH 4 - 5 was also recorded by using similar zeta-potential measurement (Iwanaga *et al.*, 2007; Nikiforidis and Kiosseoglou, 2009).

As the pH of suspension moves away from the isoelectric point to the acidic side, oil bodies have an overall positive charge reaching a zeta-potential of +30 mV and +20 mV for crude and alkali-crude oil bodies respectively, at pH 2.

4.5. Summary of results

Oil bodies were recovered from rice bran by various method including mortar and pestle method (section 4.1.1), water-based method (section 4.1.2), alkali-based method (section 4.1.3), and enzyme-assisted method (section 4.1.4). The potential methods that could recover intact oil bodies with reasonable lipid recovery yield were water-based and alkali-based method. The chemical composition, phytochemical composition and physical properties of the recovered oil body were determined. The purification of oil bodies by washing with water, 9 M urea and alkaline solution (0.1 M

$NaHCO_3$) removed extraneous/associated protein from crude oil bodies. The alkaline solution (0.1 M $NaHCO_3$) was proved to be the most aggressive washing media. It is clear that the effective lipid concentration increased while protein concentration decreased after washing the oil bodies. However, all washing protocols did not seem to remove associated protein (oleosin), and tocochromanols and oryzanol, which are associated components of rice bran oil bodies. In contrast, free fatty acids and other phenolic compounds were removed successively after washing.

Oil bodies were observed as a small discrete organelle in the aleurone and sub-aleurone layer of brown rice. However, the isolated oil bodies were larger than they appeared *in vivo* due to the limitation of the measurement. In addition, it is possible that rice bran oil bodies are fragile and therefore, coalesce *ex vivo*.

The influence of pH on the physical stability of rice bran oil body suspension was determined using light microscopy, particle size, relative turbidity and zeta-potential measurements. It shows that at neutral pH, crude oil bodies recovered from both water and alkali-based methods were relatively small in diameter (4.4 - 4.5 μm) with relatively high turbidity and zeta-potential (negatively charged surface). Washing of oil bodies with alkaline solution resulted in a considerable increase in negatively charged surface according to the

removal of alkaline proteins and positively charged metal ions from the washed oil body surface. The pI of rice bran oil body suspension was determined by measuring zeta potential of the oil bodies suspended in buffers over a range of pH. The pI was determined at pH 4 - 5 and was dependent on the oil body recovery and washing protocol. The results from relative turbidity measurement also confirmed the same finding.

5. IMPACT OF HEAT TREATMENT AND STORAGE ON THE QUALITY OF RICE BRAN AND ITS OIL BODIES

The milling process is highly disruptive; therefore it is extremely challenging to prevent rapid chemical and physical degradation of rice bran in order to maintain its quality at the highest level. The instability of rice bran is mainly due to the relatively high levels of lipase activity in raw rice bran. In intact rice kernel, rice bran lipase is physically separated from lipid. Lipase is contained in the cross-testa layer of the kernel whereas lipid is located in the aleurone and sub-aleurone layer and germ (Luh *et al*., 1991) (Figure 5.1). Once the bran is removed from the kernel, brown rice surface is disturbed and the lipid and lipase are brought together.

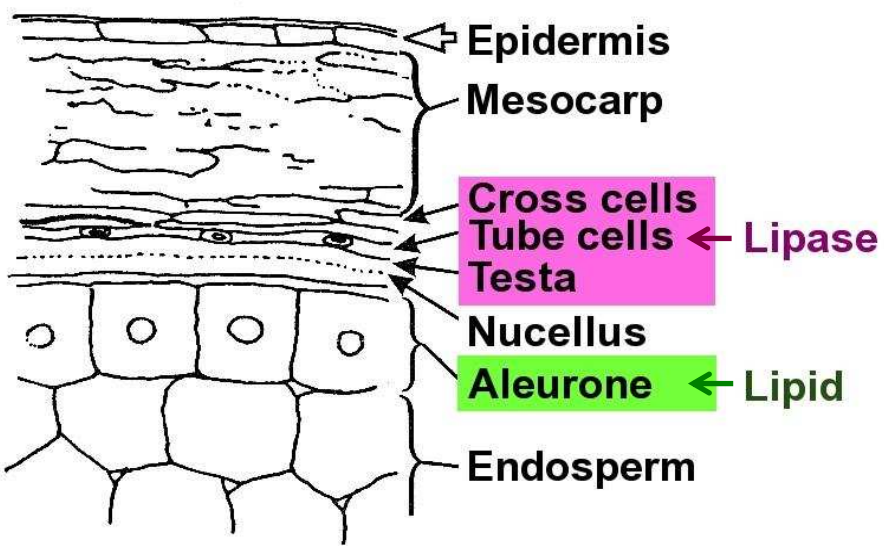


Figure 5.1 Microstructure of the outer layers of rice kernel showing the locations of lipase (cross-testa layer) and lipid (aleurone layer) (adapted from Champagne (2004))[a]

[a] Sub-aleurone layer is not presented in the Figure.

The lipid of intact bran is reported to contain 2 - 4% free fatty acids (FFAs) (Orthoefer, 1996). However, after milling, free fatty acids increases rapidly. The hydrolysis of lipid to free fatty acids results in high lipid loss during storage and oil refining problems when rice bran is not processed immediately at the place where the bran is milled. In addition, lipoxygenase and peroxidase also affect oxidative stability of rice bran. The oxidative instability is responsible for rancid flavour and off odour of rice bran (Champagne, 2004). Stabilization of rice bran is therefore required to inactivate these active enzymes.

This chapter discusses the impact of rice bran storage on quality of rice bran and its oil body. Effect of temperature alone and the effect of both temperature and humidity on rice bran during storage are compared. Morphological, chemical and physical characteristics of the stored rice bran are also included in this chapter. Stabilization of rice bran by heat treatment including hot air drying and extrusion cooking were performed to study their impact of the stabilization on characteristics of rice bran and oil bodies recovered from the heat-treated rice bran.

5.1. Effect of temperature on rice bran during storage

5.1.1. Basic composition

Freshly milled bran in this study derived from brown rice grains that had been stored for more than one year. The fresh rice bran was placed on aluminium trays and stored in an incubator (Sanyo MIR-153, UK) at 4 ºC, 37 ºC and 45 ºC without controlling relative humidity for 60 days. Rice bran (about 50 g) was taken out on day 0, 5 and subsequently every 10 days. The stored samples were kept in a freezer at -80 ºC until analysis. The three different temperatures were selected to cover the optimum temperature for lipase activity (37 ºC) (Aizono *et al.*, 1973). Relative humidity (RH) in the incubator was recorded during storage, the average RH values were 82.4 ± 3.0, 36.4 ± 1.9, and 32.2 ± 0.2 % in the incubator at temperature settings of 4 ºC, 37 ºC and 45 ºC respectively.

Moisture content of rice bran during storage is shown in Figure 5.2. Undoubtedly, a decrease of moisture content was observed during the storage because of the high temperatures (37 ºC and 45 ºC). There were no significant changes in lipid and protein content on a dry weight basis during storage (Figure 5.3 and Figure 5.4 respectively).

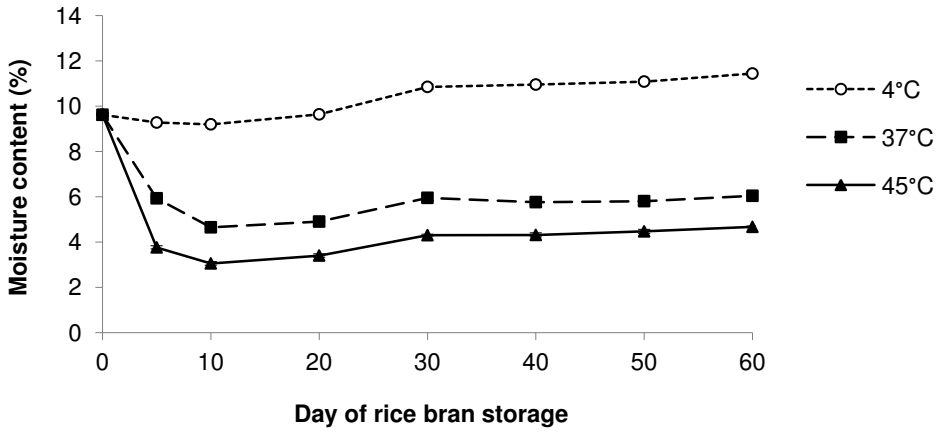


Figure 5.2 Moisture content of rice bran during storage at 4 ºC, 37 ºC and 45 ºC for 60 days

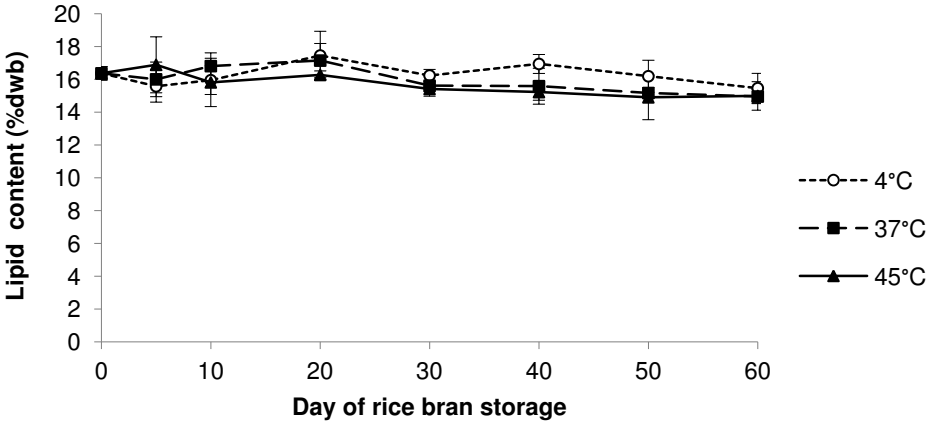


Figure 5.3 Total lipid content (% dry weight basis) of rice bran during storage at 4 ºC, 37 ºC and 45 ºC for 60 days

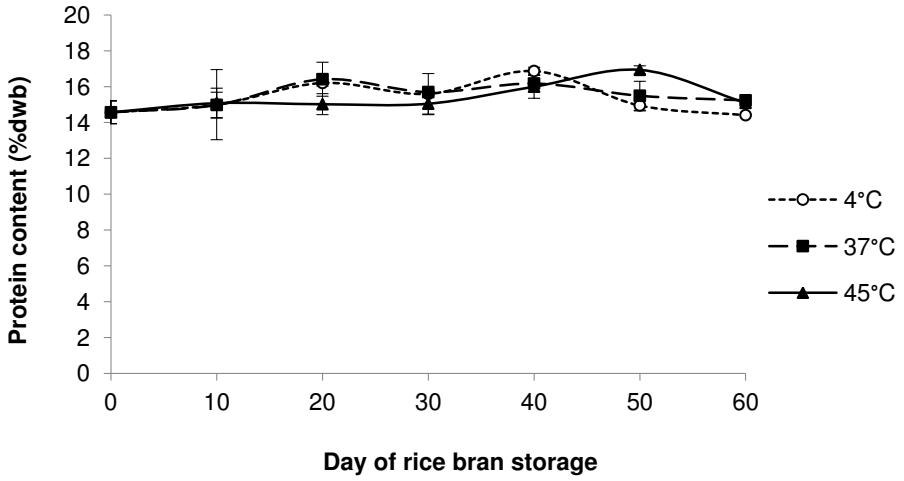


Figure 5.4 Protein content (% dry weight basis) of rice bran during storage at 4 ºC, 37 ºC and 45 ºC for 60 days

5.1.2. Phytochemical composition

Total tocochromanol and oryzanol concentrations of rice bran were measured during 60 days (Figure 5.5A). The rate of tocochromanol decrease was fastest at 45 ºC, followed by those at 37 ºC and 4 ºC respectively. The losses of total tocochromanols based on rice bran lipid basis on day 60 were 15.5 %, 33.7 % and 44.8 % at 4 ºC, 37 ºC and 45 ºC respectively. In contrast, oryzanol was relatively stable during the storage at all investigated temperatures (Figure 5.5B). The stability of oryzanol to high temperature over tocochromanols was similar to previous studies of Shin *et al.* (1997) who determined the changes of tocochromanols and oryzanol of raw and extruded rice bran during storage.

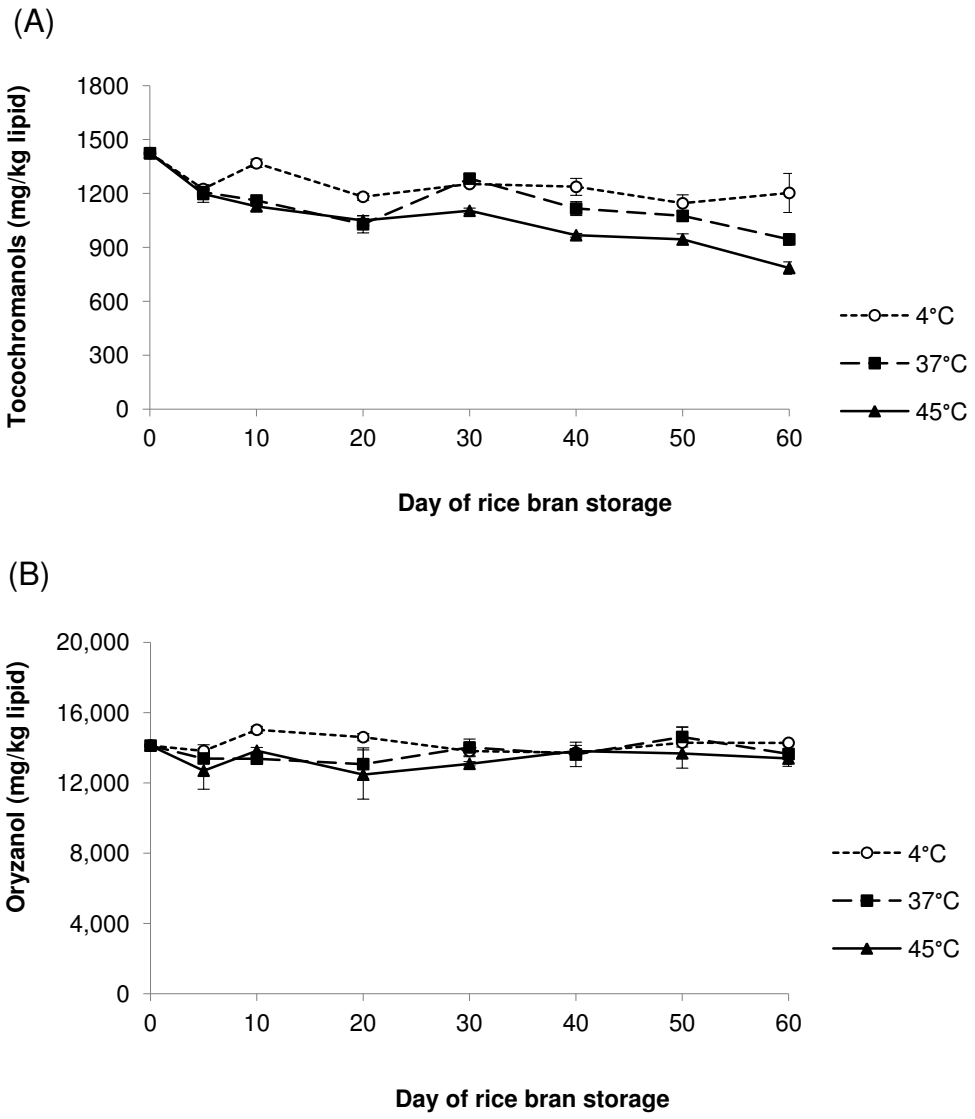


Figure 5.5 Phytochemical concentrations of rice bran during storage at 4 ºC, 37 ºC and 45 ºC for 60 days (A) total tocochromanols and (B) oryzanol

In this study, total phenolic content (TPC) of rice bran during the storage, determined using Folin-Ciocalteu reagent, is shown in Figure 5.6. Freshly milled rice bran contained 18,332 ± 4,460 mg GAE/kg lipid. It appeared that the total phenolic content significantly increased ($P<0.05$), primarily during the initial storage (until 20 days)

and then decreased gradually. However, the total phenolic content of rice bran at the end of the storage (day 60) at all temperatures was not significantly different to that of the starting value.

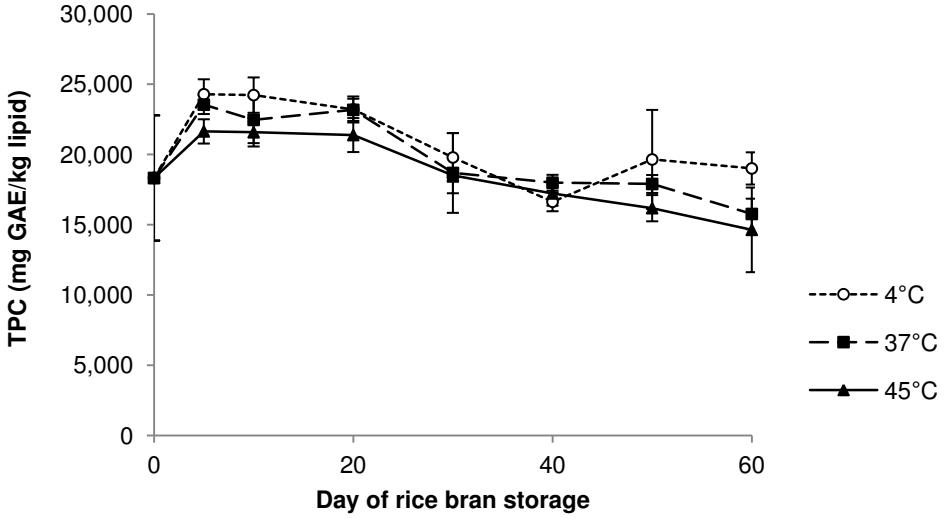


Figure 5.6 Total phenolic content of rice bran during storage at 4 ºC, 37 ºC and 45 ºC for 60 days

Results were expressed as milligram of gallic acid equivalents (GAE) per kilogram lipid in rice bran

5.1.3. Antioxidant capacity

Ferric-ion reducing antioxidant power (FRAP) assay was originally employed to assay antioxidant power in plasma by the reduction of ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous ion (Fe^{2+}) at low pH (Benzie and Strain, 1996). However, in this study, we used FRAP to measure the antioxidant capacity of rice bran during the storage because of its simplicity and rapid measurement. FRAP assay also provides an index of ability to withstand oxidation

of reactive species presented in reaction mixture. From Figure 5.7, antioxidant capacity of freshly milled rice bran is 64.4 ± 7.9 mmol/kg lipid in rice bran. The antioxidant capacity of rice bran was significantly increased ($P<0.05$) initially from day 5 to day 20 and decreased afterward. However, the antioxidant capacity of rice bran at the end of the storage was not significantly different from that of fresh rice bran. The changes of the antioxidant capacity during the storage of rice bran at all studied temperatures (4 ºC, 37 ºC and 45 ºC) were similar.

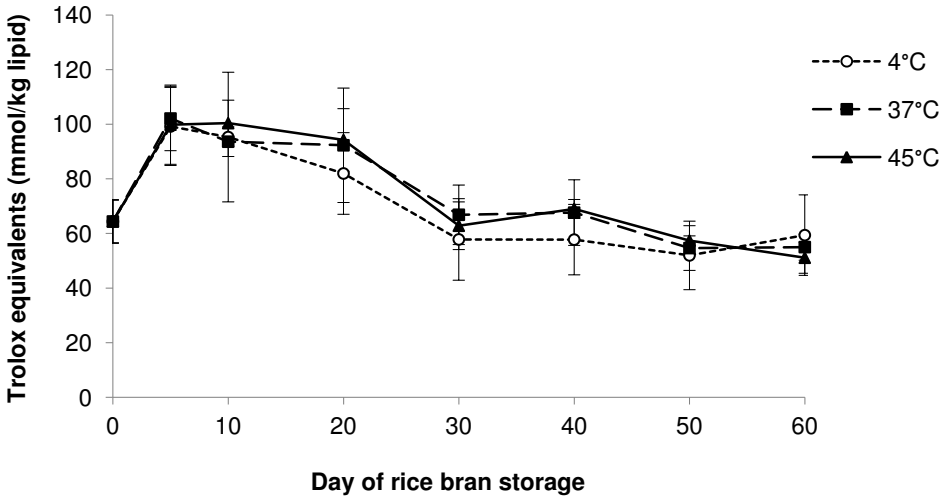


Figure 5.7 Antioxidant capacity of rice bran stored at 4 ºC, 37 ºC and 45 ºC for 60 days

Results were expressed as trolox equivalents (mmol) per kilogram lipid in rice bran

Tocotrienols, tocopherols, oryzanol and several phenolic compounds exhibit beneficial effects such as antioxidant activity and antibacterial activity against lipid peroxidation and lipid deterioration during storage. It can be seen that general trend of the antioxidant capacity change with storage time/temperature reflects the variation in the content of antioxidants (tocochromanols and oryzanol) and total phenolic content of rice bran. In addition, the total phenolic content has previously been found to positively correlate with antioxidant activities of rice bran (Aguilar-Garcia *et al.*, 2007; Goffman and Bergman, 2004). It is possible that the mechanical stress from rice milling and physical stress during the storage induce a number of physiological responses to protect rice bran cells from severe damage. An example of biological reaction against elevated temperature is heat shock response. It results in the accumulation of various secondary metabolites including phenolic compounds, such as phenylpropanoids, flavonoids and plant steroids (Ahmad and Prasad, 2011; Saltveit, 2000). These compounds act as effective antioxidants and are essential in protecting cellular structures under the stress conditions.

5.1.4. Lipid hydrolysis

After rice milling, rice bran lipids are exposed to lipases and this results in rapid hydrolysis of triacylglycerols to free fatty acids and glycerols. Figure 5.8 shows the changes of free fatty acids

(FFAs) of freshly milled rice bran during storage. The formation of free fatty acids increased dramatically after the storage at all investigated temperatures. In general, the FFA levels changed in three different phases. Firstly, the levels of FFAs significantly increased during the first 5 days. Secondly, they stabilized until around day 20 and then thirdly, they gradually increased until the end of the storage. The FFA levels increased with increasing temperature of the storage between day 40 and 60.

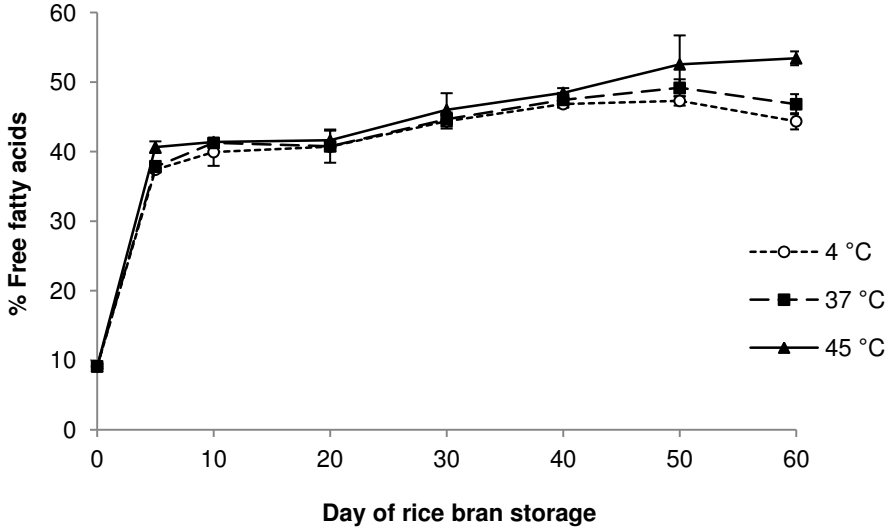


Figure 5.8 Free fatty acid content of fresh rice bran stored at 4 ºC, 37 ºC and 45 ºC for 60 days

Results were calculated as oleic acid and expressed as a percentage (% w/w) of the total lipid content in rice bran

The rapid formation of FFAs during the early storage could be explained by activity of rice bran lipase. The static levels of FFAs during the second phase of the storage trial was related to product inhibition or equilibrium between the FFA formation and oxidative degradation (Lam and Proctor, 2003). Although the optimal temperature for the action of rice bran lipase is at 37 ºC, the highest level of FFA was produced at 45 ºC. This suggests that not only rice bran lipase, but also other lipases (such as microbial lipases) are responsible for the increase of FFA level since the optimal temperatures of microbial lipases can be active around 45 ºC (Ghosh *et al.*, 1996). In addition to lipase, rice bran also contains lipoxygenase and peroxidase (Champagne, 2004). These enzymes have been reported to have a negative impact on oxidative stability of rice bran. The oxidative deterioration is responsible for the change of flavour and aroma of rice bran.

5.1.5. Oxidative degradation

Oxidation of lipids in rice bran during storage was measured by lipid hydroperoxide concentration (Figure 5.9). The ferric thiocyanate method for lipid hydroperoxide value, based on the oxidation of ferrous to ferric ions, is more sensitive and requires a smaller amount of sample than standard iodometric method measured by titration (Frankel, 2005). The changes of lipid hydroperoxide followed a similar trend of free fatty acid formation

(Figure 5.8). The FFA level and hydroperoxide concentration increased rapidly after rice milling, but the increased rates were not dependent on temperature during the first 5 days. Between day 10 and day 30, the level of both FFAs and hydroperoxides were highest at 45 ºC. The level of FFAs continued to increase most rapidly at 45 ºC until the end of the storage. In contrast, the concentrations of lipid hydroperoxide at 45 ºC leveled off after 30 days and at 25 ºC reached the peak on day 40. The decrease of lipid hydroperoxide could be attributed to break down of the hydroperoxide to secondary oxidation products (Frankel, 2005). However, determination of secondary oxidation products was not performed in this experiment.

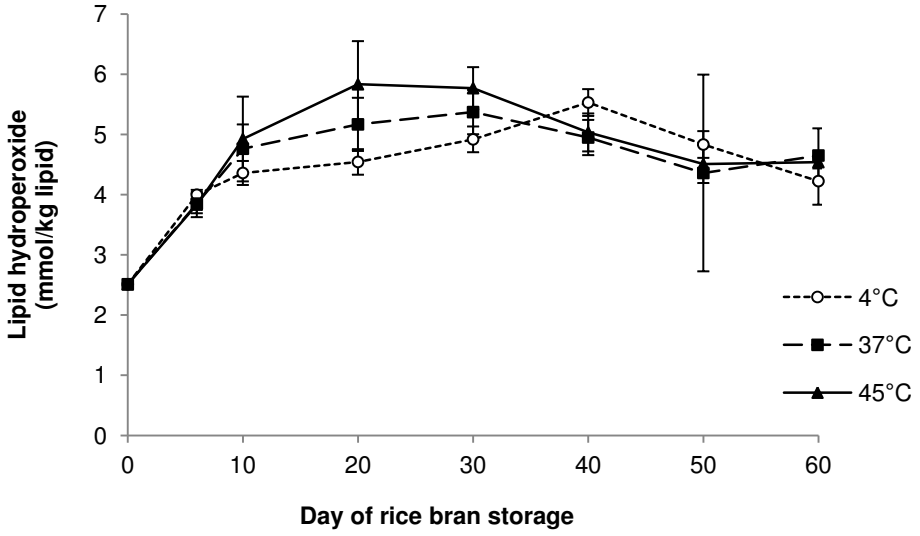


Figure 5.9 Lipid hydroperoxide concentration of rice bran stored at 4 ºC, 37 ºC and 45 ºC for 60 days

Results were expressed as mmol of cumene hydroperoxide per kg total lipid content in rice bran

Some fluctuations in hydroperoxide concentrations were observed during the storage as the measurements were taken in days not in minutes or hours. It is possible that a peak of the hydroperoxide concentration was missed. The concentration of hydroperoxides of 2.5 mmol/kg lipid was reported as a limit of acceptability for polyunsaturated vegetable bulk oil (Frankel, 2005). The hydroperoxide concentrations of oil extracted from rice bran after the storage trial for a few days were higher than the specified level; therefore, the extracted oil could be classified as poor quality oil.

5.2. Effect of temperature and moisture on rice bran during storage

Freshly milled rice bran was placed on aluminium trays and stored in a humidity chamber (Binder KBF series, USA) at 25 ºC and 45 ºC with 75 % relative humidity for 40 days. Rice bran (about 50 g) was taken out on day 0, 5 and subsequently every 10 days. The stored samples were kept in a freezer at -80 ºC until analysis. The experiment was set to evaluate the changes of rice bran and the oxidative deterioration of rice bran lipid under the accelerated storage conditions (45 ºC with 75 % relative humidity) compared with the control (25 ºC with 75 % relative humidity). After storing for 50 days, yellow-green spots and mold hyphae were observed in the samples, especially rice bran stored at 45 ºC. The storage was thus terminated.

5.2.1. Imaging of stored rice bran

Transmission electron micrographs of stored rice bran on day 40 at 45 ºC and 75 % relative humidity (Figure 5.10) show large oil body droplets (2 - 4 µm), often with angular shapes and a loss of surface integrity.

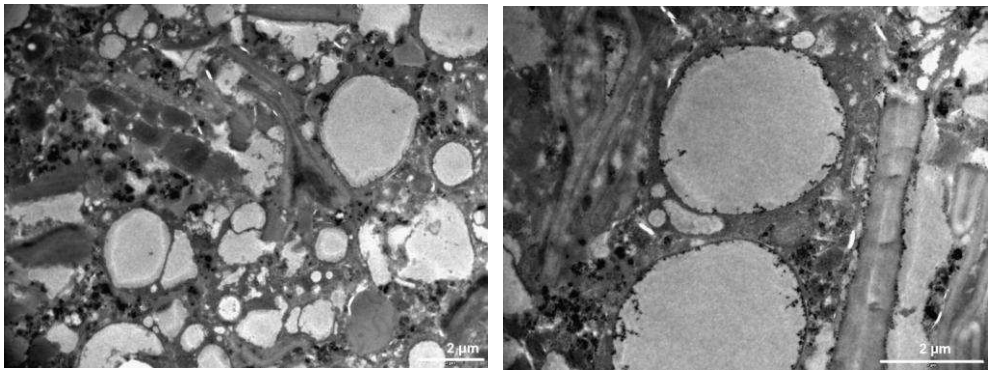


Figure 5.10 Transmission electron micrographs of stored rice bran at 45 ºC and 75 % relative humidity on day 40

5.2.2. Basic composition

Rice is a hygroscopic material. When environment of the rice grains is changed, they absorb or desorb moisture (Champagne, 2004). Figure 5.11 shows moisture content of rice bran during storage at different temperatures (25 ºC and 45 ºC) under controlled relative humidity (75 %). The storage of rice bran at the high relative humidity resulted in an increase of moisture content during the first five days of storage before returning to near original values on day

10 and then attaining equilibrium until day 20. The decrease of moisture content after day 5 to day 10 may be due to the use of water in the metabolism of rice bran such as lipid hydrolysis, or changes within rice bran material that caused a loss of water from starch granules. After day 20, the moisture content of rice bran increased again probably because of the growth of microorganisms and their generation of water from respiration.

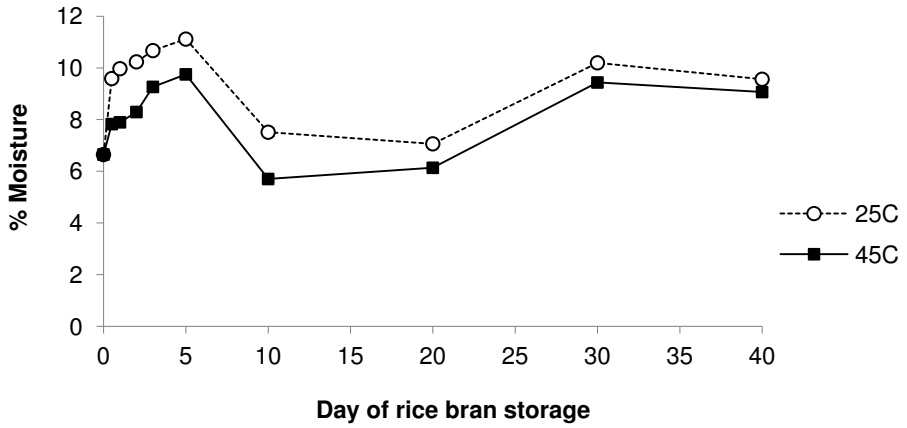


Figure 5.11 Moisture content of rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

At 75 % relative humidity, the moisture content of rice bran stored at 25 ºC was higher than that of 45 ºC. Because the fact that water evaporates faster at the high temperature than at the low one, the absorbed water or moisture content of the bran stored at 45 ºC was thus lower.

There was no significant change in total lipid content of rice bran during the storage (Figure 5.12). However, some fluctuations in protein content were observed (Figure 5.13). In addition, the total dry mass (sum of lipid and protein content) of rice bran during the storage was not stable. It may be attributed to changes of physical structure within rice bran under the storage conditions (for example, cross-linking formation between starch-protein that reduced the protein extractability; cross-linking formation between protein-protein as a result of lipid oxidation that caused changes of the primary structure of protein and the protein measurement by BCA method). Furthermore, the increase of total dry mass and protein may be related to the growth and enzyme production from microorganisms.

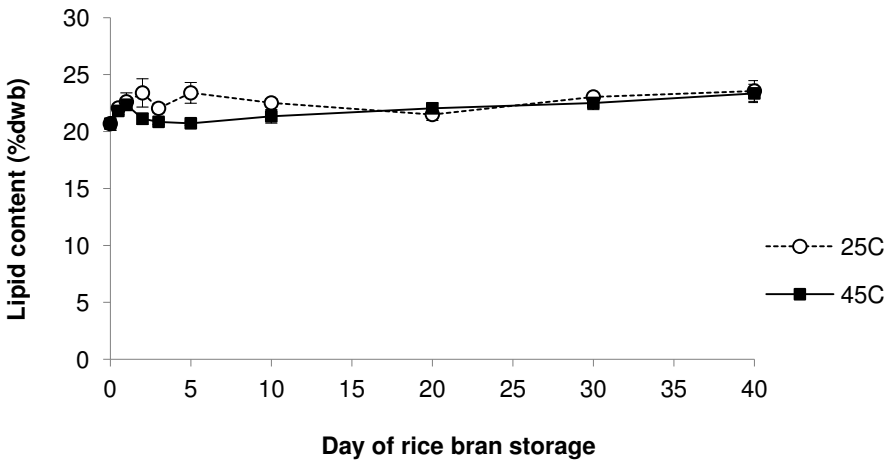


Figure 5.12 Lipid content (% dry weight basis) of rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

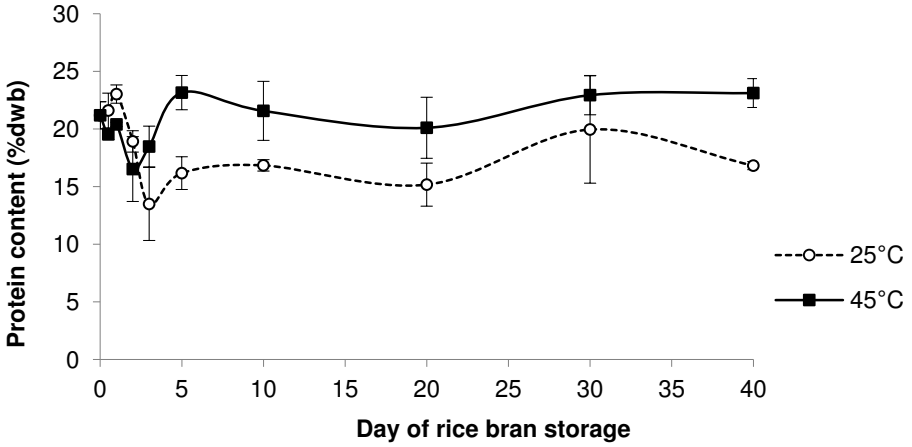


Figure 5.13 Protein content (% dry weight basis) of rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

5.2.3. Water activity

The water activity of rice bran was measured as an indicator of the storage stability of foods with low moisture content. Increased water activity promotes the growth of microorganisms and hastens enzymatic reactions (specifically involving hydrolases) (Belitz *et al.*, 2004). The water activity was measured as described in section 2.8. After transferring freshly milled rice bran (a_w 0.42) to the tested conditions, rice bran absorbed moisture from the surrounding air which resulted in an increase of water activity until day 10 and then attained equilibrium until the end of the storage (a_w 0.68 and a_w 0.65 at 25 ºC and 45 ºC on day 40 respectively) (Figure 5.14).

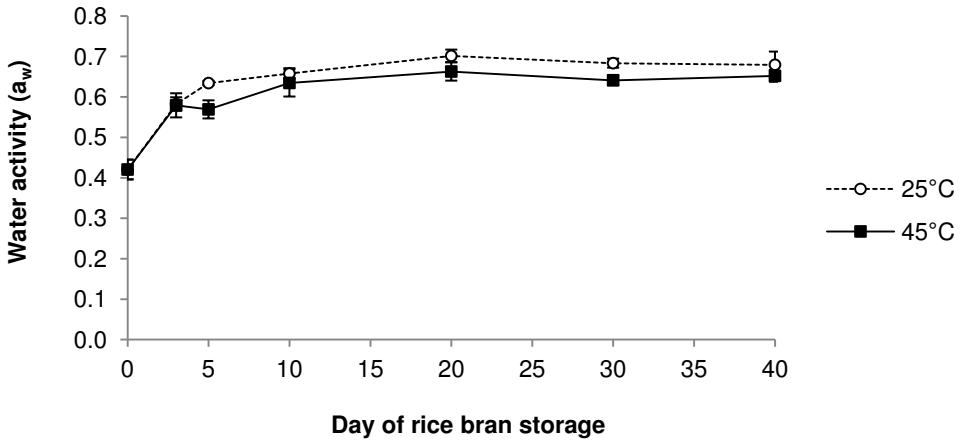


Figure 5.14 Water activity (a_w) of rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

5.2.4. Fatty acid composition

Fatty acid composition of total lipids of rice bran stored at 25 ºC and 45 ºC is compared in Figure 5.15. At both temperatures, the concentrations of both saturated fatty acids (palmitic and stearic acid) and unsaturated fatty acids (oleic, linoleic and linolenic acid) changed very slightly. Although the fatty acid composition was statistically significant ($P<0.05$), the differences were negligible.

5.2.5. Phytochemical composition

Total tocochromanol and oryzanol levels of rice bran were measured during 40 days of the storage (Figure 5.16). The rates of tocochromanol decrease in rice bran stored at 25 ºC and 45 ºC were similar. The losses of total tocochromanols based on lipid basis on

day 40 relative to the original values were 17.4 % and 17.6 % at 25 ºC and 45 ºC respectively. In contrast, oryzanol was relatively stable during the storage at all investigated temperatures (Figure 5.16B).

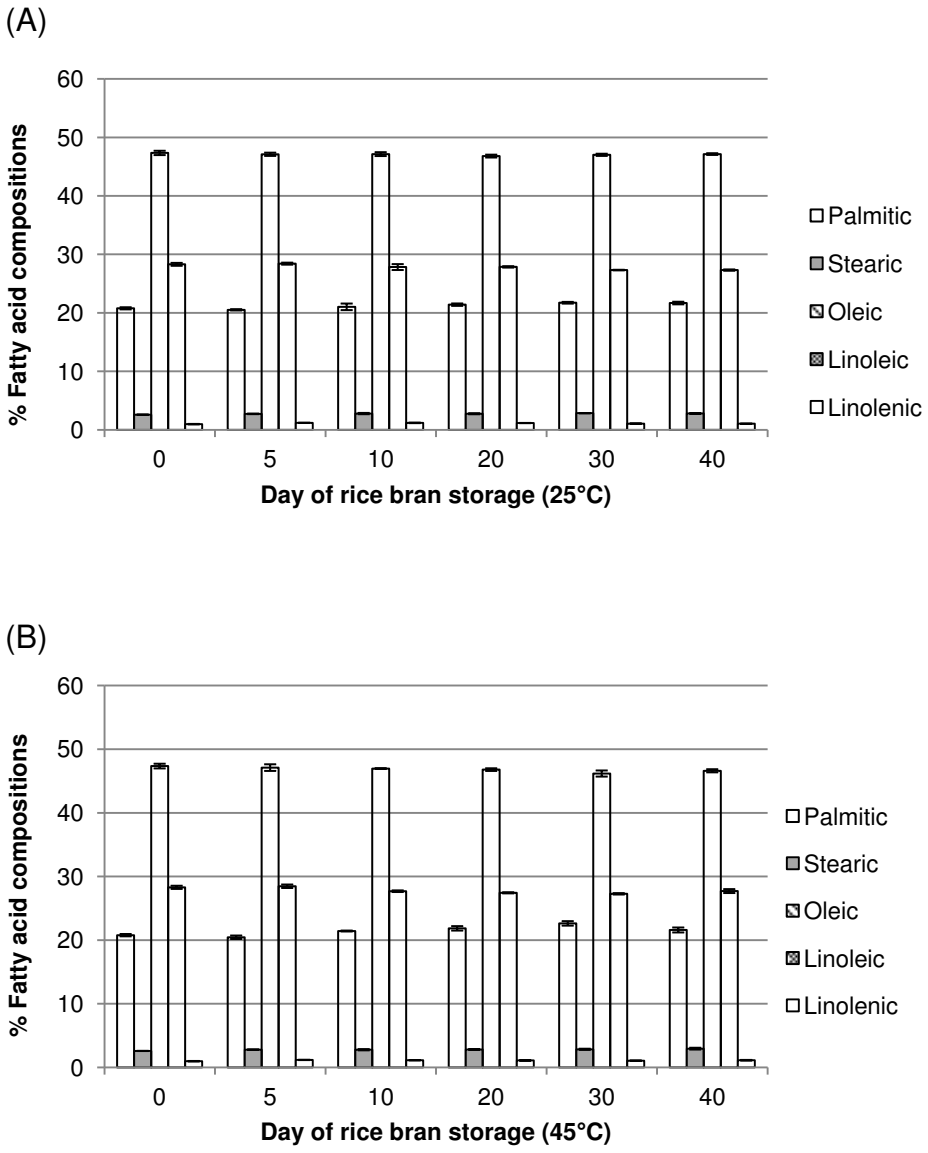


Figure 5.15 Fatty acid composition of total lipid extracts of rice bran during storage at (A) 25 ºC and (B) 45 ºC and 75 % relative humidity for 40 days

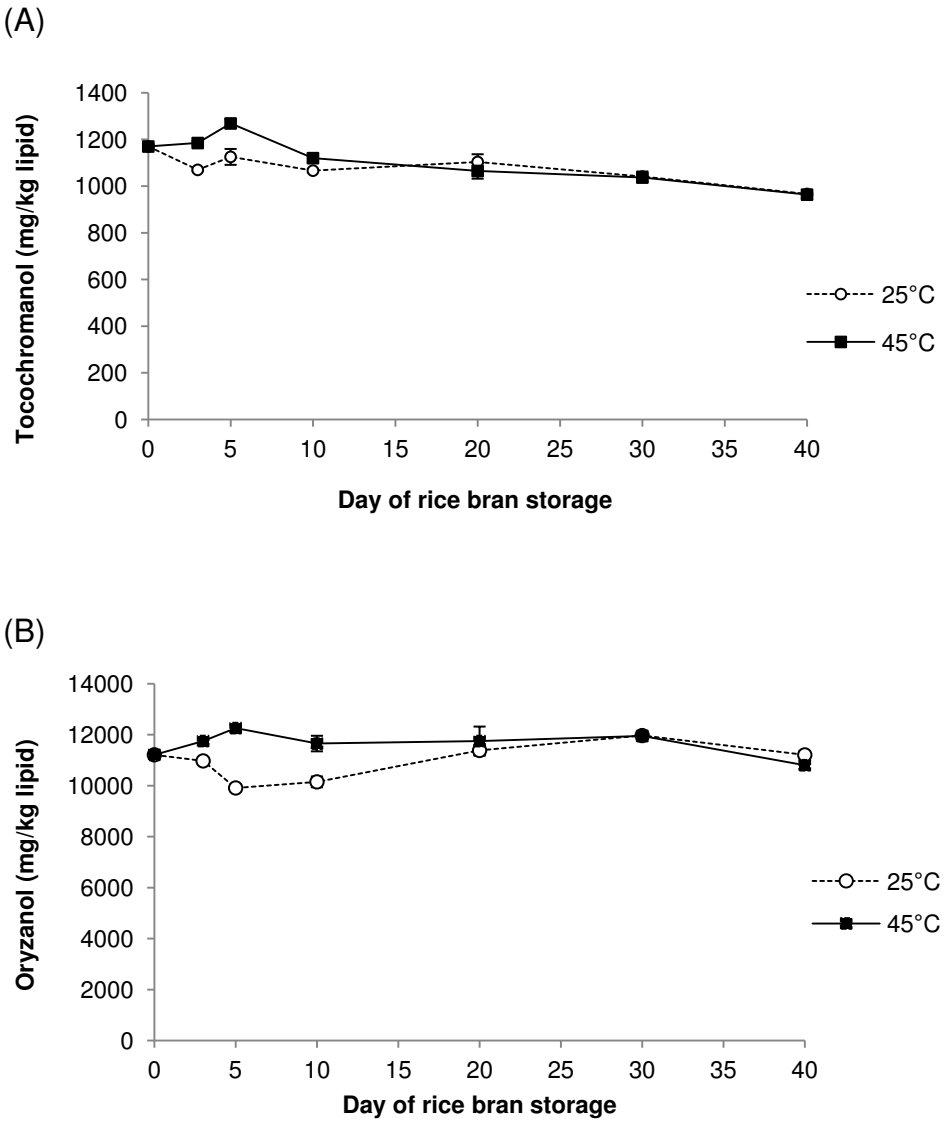


Figure 5.16 Phytochemical concentrations of rice bran (A) total tocochromanols and (B) oryzanol during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

Total phenolic content (TPC) of rice bran during the storage is shown in Figure 5.17. Freshly milled rice bran contained TPC 12,096 ± 464 mg GAE/kg lipid. The TPC decreased gradually during the storage. The losses of TPC of rice bran on day 40 were 16.7 % and 14.7 % at 25 ºC and 45 ºC respectively.

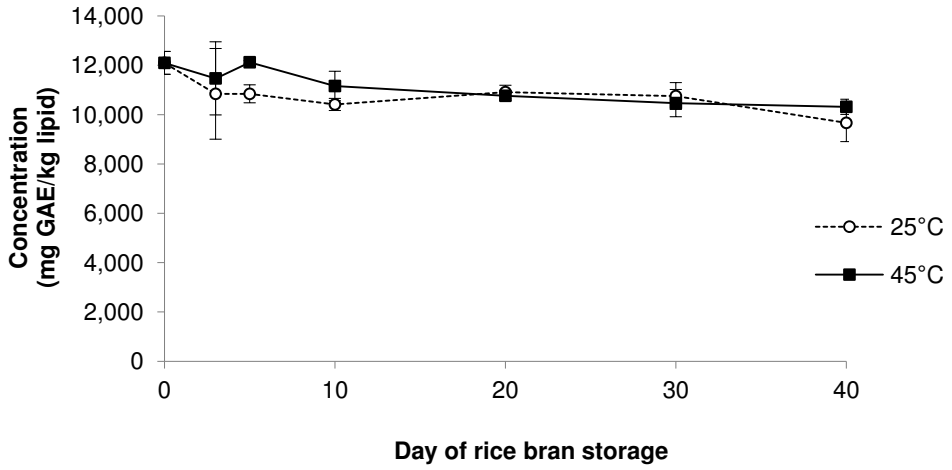


Figure 5.17 Total phenolic content of rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

5.2.6. Antioxidant capacity

From Figure 5.18, antioxidant activity of freshly milled rice bran is 47.8 ± 5.1 mmol/kg lipid in rice bran. The antioxidant activity of rice bran decreased after storage. Similar changes of the antioxidant activity were observed at both temperatures (25 ºC and 45 ºC).

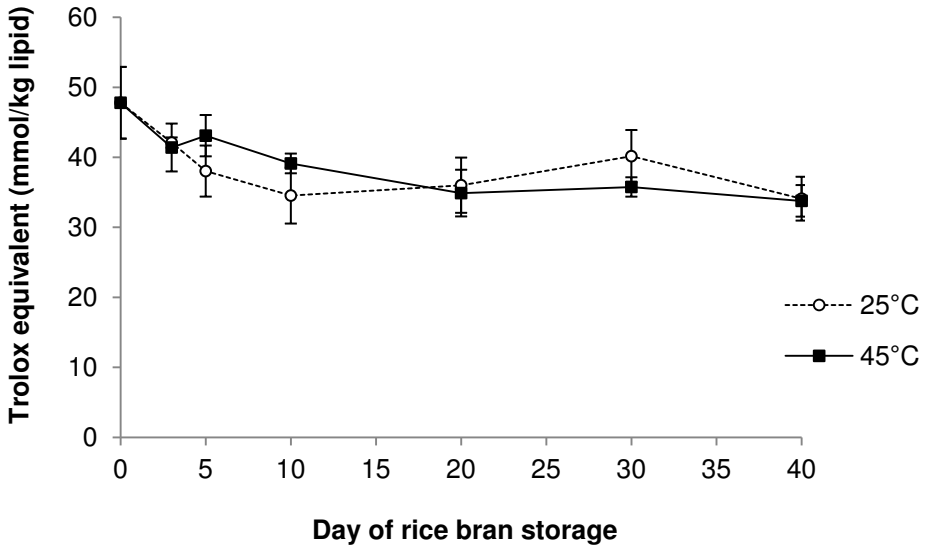


Figure 5.18 Antioxidant capacity of rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

5.2.7. Free fatty acid formation

The major lipid in freshly milled rice bran is triacylglycerols (lane 1, Figure 5.19). The amount of triacylglycerols in rice bran lipids decreased while that of free fatty acids increased during the storage at both temperatures. Indeed, the triacylglycerol reduction was higher in rice bran stored at 45 ºC (Figure 5.20) than at 25 ºC. This indicated that lipase activity was higher in rice bran stored at 45 ºC than at 25 ºC.

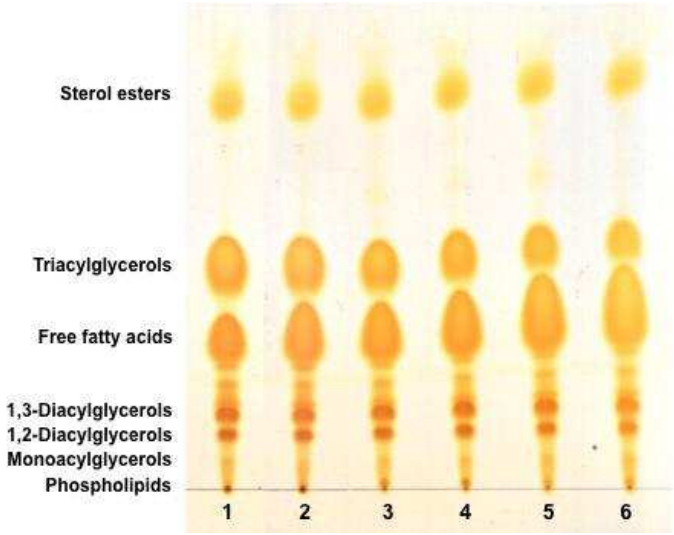


Figure 5.19 Separation of stored (25 ºC and 75 % relative humidity) rice bran lipid classes on TLC plate (Silica Gel 60)

Developing solvent: hexane-diethyl ether-acetic acid (80:20:2 by volume). 1, fresh rice bran; 2, rice bran stored 5 days at 25 ºC; 3, 10 days at 25 ºC; 4, 20 days at 25 ºC; 5, 30 days at 25 ºC and 6, 40 days at 25 ºC

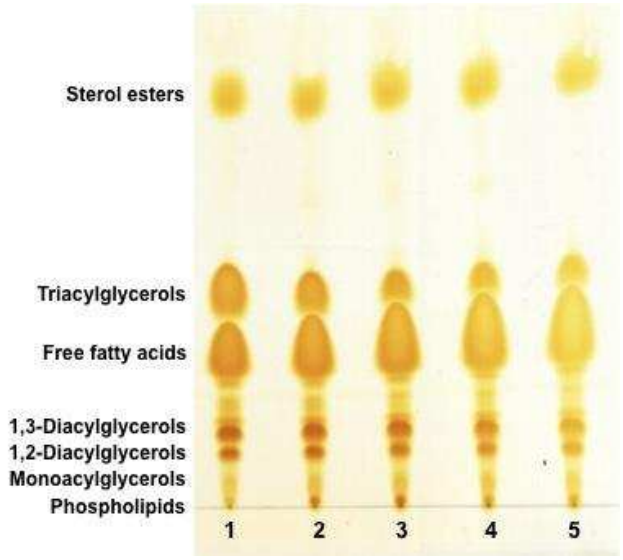


Figure 5.20 Separation of stored (45 ºC and 75 % relative humidity) rice bran lipid classes on TLC plate (Silica Gel 60)

Developing solvent: hexane-diethyl ether-acetic acid (80:20:2 by volume). 1, rice bran stored 5 days at 45 ºC; 2, rice bran stored 10 days at 45 ºC; 3, 20 days at 45 ºC; 4, 30 days at 45 ºC; and 5, 40 days at 45 ºC

Analysis of free fatty acids of rice bran lipids in stored rice bran at controlled 75 % relative humidity (Figure 5.21) confirmed this observation. The levels of FFA rapidly increased after milling until day 10, then slowly increased until day 20 and dramatically increased until the end of the storage. As compared with rice bran stored at the uncontrolled and low relative humidity (measured as 32 %) at 45 ºC (Figure 5.8), the levels of FFA of rice bran stored at the high relative humidity (75 %, Figure 5.21) increased at a much higher rate. This shows that the levels of FFA varied with temperature and relative humidity during the storage.

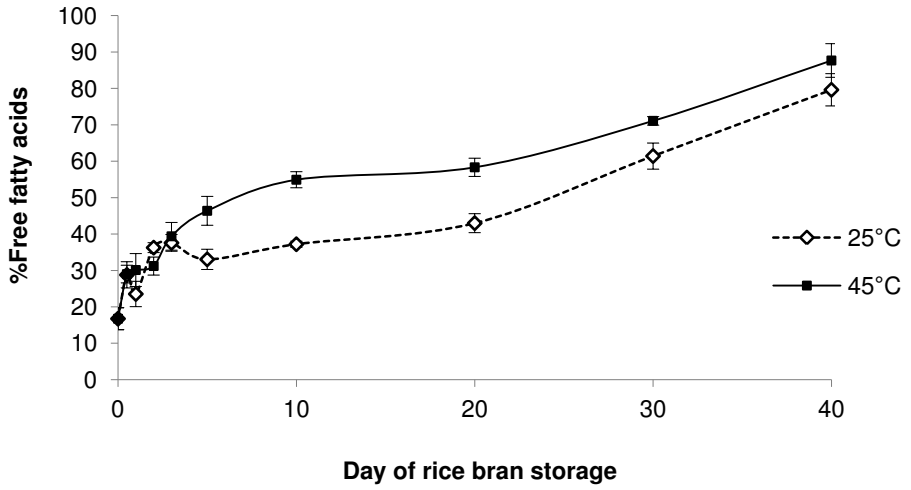


Figure 5.21 Free fatty acid content of rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

Results were calculated as oleic acid and expressed as a percentage (% w/w) of the total lipid content in rice bran

5.2.8. Lipid oxidation

Lipid oxidation is initiated by enzymatic and non-enzymatic (autoxidation) pathways. Unsaturated FFAs accumulated immediately after milling are a major source for the lipid oxidation catalyzed by lipoxygenases. The reaction generates lipid hydroperoxides that further decompose to volatiles and non-volatile components. These breakdown products are responsible for changes of flavour, colour and nutritive properties. The primary oxidation product (lipid hydroperoxides) and volatile (hexanal) from the secondary oxidation product were determined to assess the oxidative stability of rice bran lipids during the storage.

Concentrations of lipid hydroperoxide were significantly increased ($P<0.05$) after milling and leveled off after 10 days (Figure 5.22). The formation of lipid hydroperoxide was more pronounced in rice bran stored at the high temperature. In addition, the maximum level of lipid hydroperoxides produced under the controlled and high relative humidity (75 %) was almost three times as much as the level at the uncontrolled and low relative humidity (Figure 5.9). This can be explained by the increased mobilization of catalysts (such as enzymes involved in the oxidation and trace metals) to the lipid-water interface at high water activity (a_w 0.7), thus facilitating the lipid oxidation.

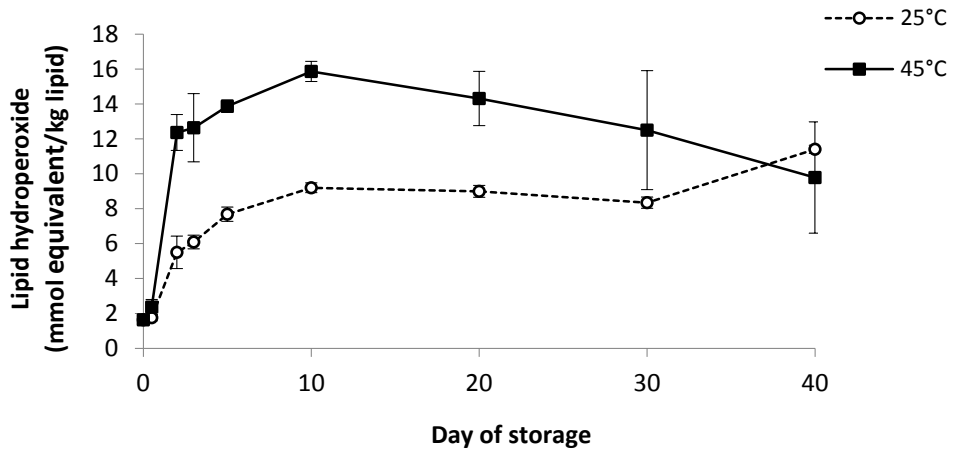


Figure 5.22 Lipid hydroperoxide concentration of rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

Results were expressed as mmol of cumene hydroperoxide equivalent per kg total lipid content in rice bran

The breakdown of lipid hydroperoxides produces aldehyde, ketones, alcohols, hydrocarbon, esters, furans, and lactones (Frankel, 2005). These volatiles were measured by solid-phase microextraction (SPME). Hexanal was used as a marker for the secondary oxidation since it was easily detectable, derived from linoleate hydroperoxides (an oxidation product from one of the major unsaturated fatty acid in rice bran) and significantly correlated with the oxidation (Frankel, 2005).

Figure 5.23 shows the change of hexanal during rice bran storage. The hexanal level increased gradually and reached a peak at day 20 and day 30 in rice bran stored at 45 ºC and 25 ºC respectively. Indeed, the total amount of hexanal in rice bran stored

at 45 ºC was lower than at 25 ºC. It was possible that at the high temperature, the lipid hydroperoxides reacted with other materials and rapidly decomposed to other compounds or the hexanal was not stable and reacted with other materials or was oxidized to product such as hexanoic acid.

Overall, the oxidation data shows that rice bran was extremely unstable to oxidation over the storage and especially at the elevated temperature and humidity.

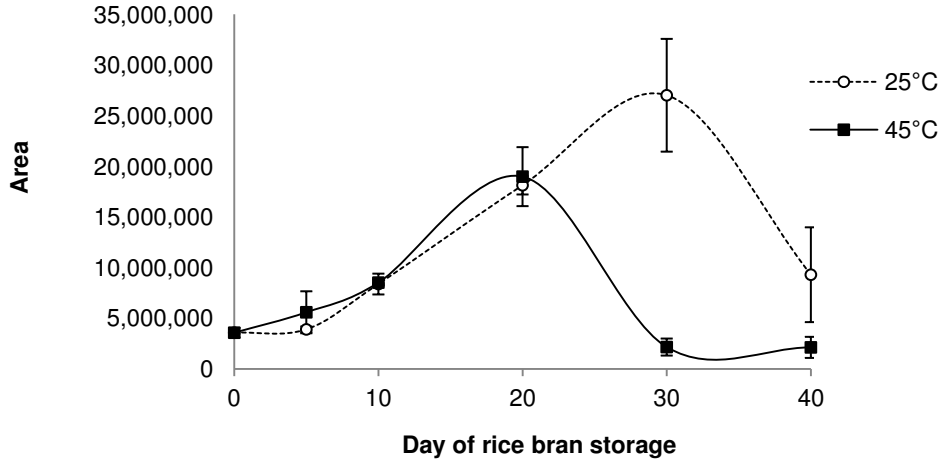


Figure 5.23 The change of hexanal in rice bran during storage at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

5.3. Effect of rice bran storage on oil body characteristics

Oil bodies are naturally pre-emulsified within the seed, which may help in preventing the stored oil from oxidation. To determine the protection of oil within oil bodies in rice bran during storage, oil bodies were recovered from rice bran stored at 25 ºC and 45 ºC and 75 % relative humidity for 40 days (Section 5.2) and characterized. If oil bodies in rice bran remained intact and "unspoilt" by lipid hydrolysis or oxidation during storage, this could lead to an innovative approach for recovering rice bran oil enriched in phytonutrients from deteriorated rice bran.

5.3.1. Imaging

Light micrographs of oil bodies recovered from the stored rice bran are shown in Figure 5.24. Oil bodies can be recovered from the stored rice bran; however, aggregation and coalescence were observed. The recovered oil bodies from rice bran that had been stored showed large duplex formations. These results indicated that oil bodies could be recovered from the stored rice bran; however, the oil bodies were not intact and were largely damaged.

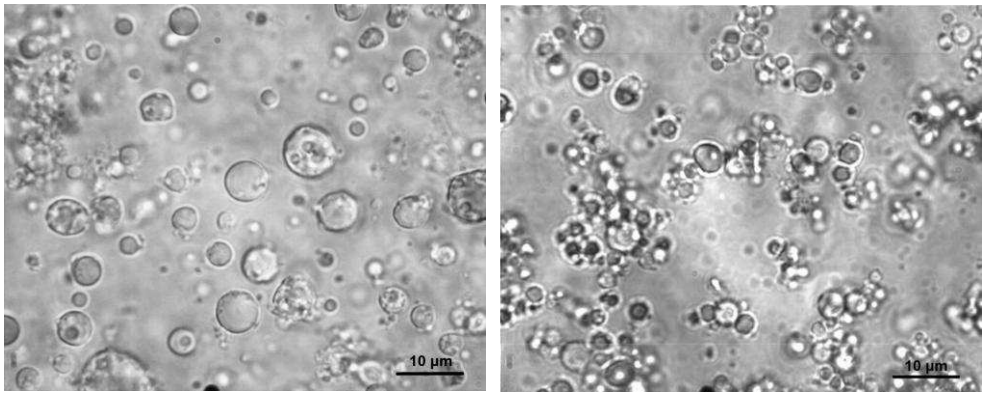


Figure 5.24 Light micrographs of oil bodies recovered from stored rice bran at 75 % relative humidity for 40 days

5.3.2. Particle size of oil bodies

Volume mean diameters D(4,3) of the oil bodies recovered from stored rice bran are shown in Figure 5.25. The results showed that the size of oil bodies changed greatly depending on the time and temperature during the storage trial. The size of oil bodies recovered from rice bran stored at 45 ºC increased dramatically until day 20 due to aggregation and coalescence and then decreased because of the degradation of oil bodies. The change in size of the oil bodies from rice bran stored at 25 ºC was similar to that of 45 ºC but to a lesser extent. In addition, Table 5.1 shows the oil body particle size by various approaches in order to describe the distribution of the samples.

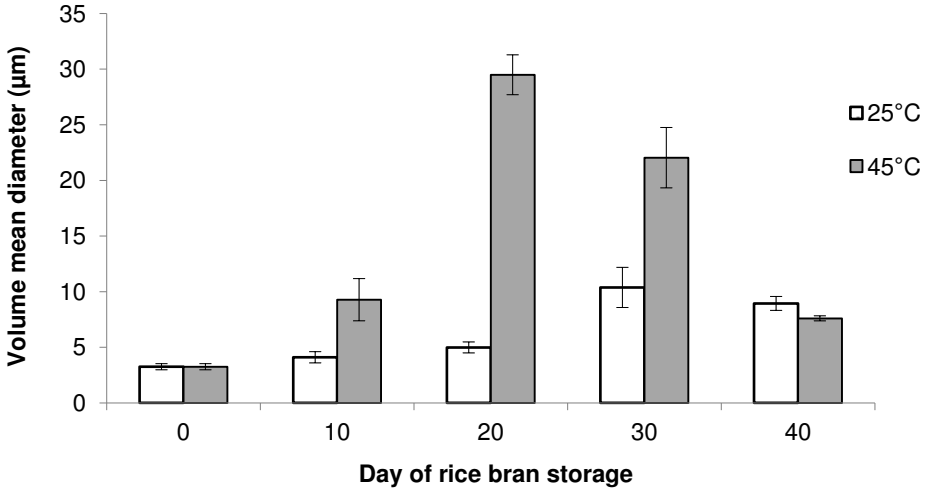


Figure 5.25 Volume mean diameters D(4,3) of oil bodies recovered from stored rice bran at 75 % relative humidity for 40 days

Table 5.1 Particle size of oil bodies recovered from stored rice bran by various approaches

Day of storage	Temperature (ºC)	D(4,3)	D(3,2)	Dv50
0	-	3.2 ± 0.3	1.9 ± 0.0	2.3 ± 0.0
10	25	4.1 ± 0.5	1.9 ± 0.0	2.6 ± 0.2
	45	9.3 ± 1.9	2.5 ± 0.1	3.8 ± 0.3
20	25	5.0 ± 0.5	1.9 ± 0.1	2.6 ± 0.1
	45	29.5 ± 1.8	5.6 ± 0.1	18.5 ± 1.0
30	25	10.4 ± 1.8	2.4 ± 0.1	4.0 ± 0.5
	45	22.0 ± 2.7	3.7 ± 0.3	10.7 ± 1.9
40	25	8.9 ± 0.6	2.5 ± 0.1	3.9 ± 0.2
	45	7.6 ± 0.2	2.4 ± 0.0	3.6 ± 0.0

5.3.3. Basic composition

Table 5.2 shows chemical composition and oil body lipid recovery of oil bodies recovered from the stored rice bran. The lipid content of oil bodies at 25 ºC decreased slightly but was statistically different ($P<0.05$) from day 30. The oil body lipid recovery yield

decreased significantly ($P<0.05$) after day 20 at 25 ºC. In comparison, only trace amount of oil bodies could be recovered from rice bran stored at 45 ºC from day 20. Therefore, measurements were not determined from these samples and were noted as ND.

Table 5.2 Chemical composition and lipid recovery yield (% dry weight basis) of oil body oil bodies recovered from stored rice bran [1,2]

Day of storage	Temperature (ºC)	Lipid [3] (% dwb)	Lipid recovery [4] (% dwb)	Protein (% dwb)
0	-	84.7 ± 3.0 [a]	28.9 ± 2.8 [a]	9.9 ± 0.3 [b]
10	25	87.0 ± 5.8 [a]	27.5 ± 1.0 [ab]	4.7 ± 0.1 [d]
	45	67.8 ± 1.4 [b]	8.2 ± 0.3 [c]	13.0 ± 1.2 [a]
20	25	84.9 ± 3.4 [a]	23.8 ± 4.3 [b]	7.1 ± 0.5 [c]
	45	ND	ND	ND
30	25	73.2 ± 2.8 [b]	9.0 ± 1.3 [c]	10.2 ± 0.4 [b]
	45	ND	ND	ND
40	25	71.7 ± 2.3 [b]	2.2 ± 0.3 [d]	12.2 ± 0.8 [a]
	45	ND	ND	ND

[1] Rice bran at 25 ºC and 45 ºC and 75 % relative humidity for 40 days.
[2] Values within columns followed by the different letter are significantly different ($p>0.05$, ANOVA) ($n = 3$, ± SD), ND = not determined
[3] Lipid content (%dwb) = lipid dry weight (g)/oil body dry weight (g) x 100
[4] Lipid recovery (%dwb) = total lipid in oil bodies (g)/total lipid in rice bran (g) x100

5.3.4. Protein composition

Gel electrophoresis of the protein fraction arising from oil bodies recovered from rice bran during storage over different times and temperatures revealed similar protein profiles (Figure 5.26). The protein with the mass of 18 kDa (band I) and 16 kDa (band J) were presumed to be oleosins (section 4.2.2.). The density of oleosin bands remained the same until day 10 at both storage temperatures.

However, there was a relative decrease in the band density of band H, I and J from day 20; and a relative increase in the band density of small molecular weight protein (band L), especially on day 30 and 40. The degradation of oleosins and other associated proteins to small protein fractions during storage may have contributed to the loss of steric hindrance that stabilized the oil bodies leading to the aggregation of oil droplets.

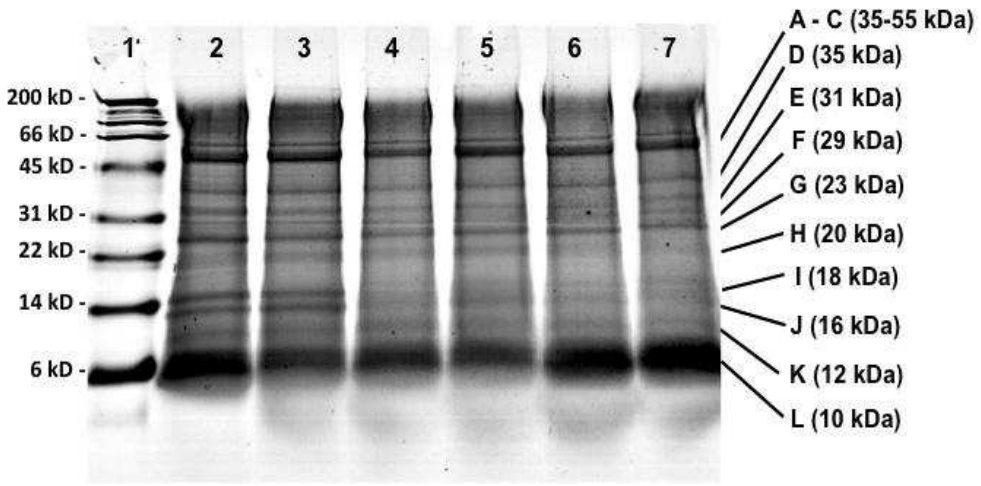


Figure 5.26 SDS-PAGE profiles in oil body preparations from stored rice bran

Lane 1, molecular weight marker; lane 2, fresh rice bran; lane 3, rice bran stored 10 days at 25 ºC; lane 4, 10 days at 45 ºC; lane 5, 20 days at 25 ºC; lane 6, 30 days at 25 ºC; and lane 7, 40 days at 25 ºC

Due to strong interaction between lipids and protein in food system, oxidation of protein and lipid can occur concurrently. The oxidation can easily transfer from lipids to protein or vice versa. The oxidation of protein is catalyzed by presence of peroxyl radicals from lipid oxidation or free metal ions such as Fe^{2+} contaminated from the

rice mill. This leads to the formation of reactive oxygen species (hydroxyl radicals, ferryl ions, perferryl ions, peroxyl radicals) and carbonyl derivatives (Levine *et al.*, 1990; Stadtman, 1992). Therefore, the low molecular weight protein fractions of oil bodies may have arisen as secondary products of the lipid oxidation.

5.3.5. Fatty acid composition

While the change in fatty acid composition rice bran was not observed in the direct solvent extraction during storage, oil bodies recovered from the same bran material displayed the change during storage. The fatty acid compositions of the total lipid extracts from oil bodies recovered from rice bran stored at 25 ºC and 45 ºC are shown in Figure 5.27A and Figure 5.27B respectively. The change in fatty acid composition became increasing apparently with increase storage time and temperature. Although saturated fatty acids (palmitic and stearic acid) represent only 18 % of the total lipids in recovered oil bodies, the saturated fatty acid reduced significantly ($P<0.05$) up to 44 % of the original value after storage for 40 days. In contrast, the unsaturated fatty acid (oleic and linoleic acid) significantly increased ($P<0.05$) by about 10 %. The result of fatty acid composition indicated that rice bran lipases preferentially cleaved saturated fatty acids than other fatty acids. Takano (1993) reported that rice bran lipases were site specific and cleave the 1,3-site of triacylglycerols. In rice, unsaturated fatty acids can be

presented in any of the three positions of the backbone of TAG. However, saturated fatty acids tend to be in the sn-1 and/or sn-3 positions (Glushenkova *et al.*, 1998). Therefore, the action of site 1,3 specific lipase enzyme will render a FFA pool enriched in saturated fatty acids compared with the TAG pool.

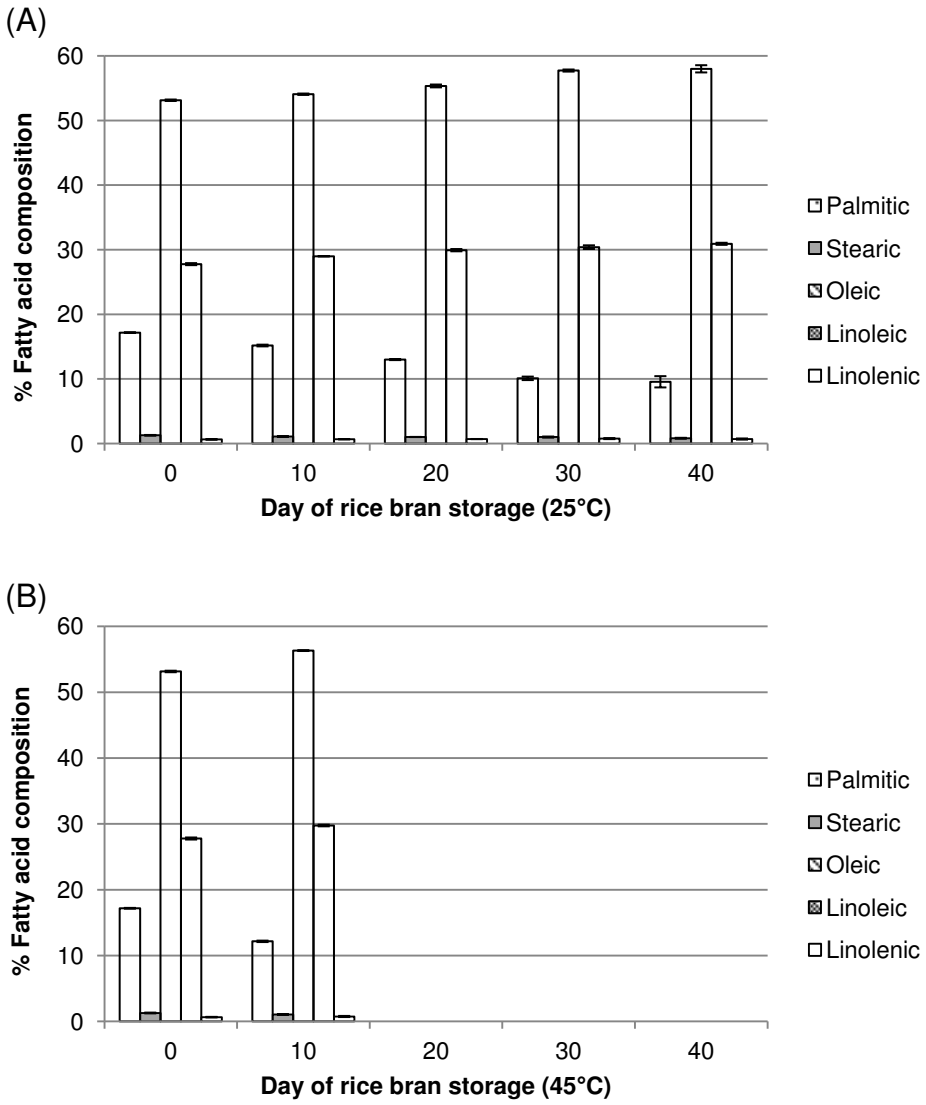


Figure 5.27 Fatty acid composition of total lipid extracts of oil bodies recovered from rice bran stored at (A) 25 ºC and (B) 45 ºC and 75 % relative humidity for 40 days

Since linolenic acid is a polyunsaturated acid; it is more prone to oxidize than other fatty acids. However, linolenic acid did not change significantly during storage at both temperatures. The stable level of linolenic acid may be related to the balance between the formation of linolenic acid from lipid hydrolysis and its degradation from lipid oxidation. In addition, the conversion of linolenic acid to lipid hydroperoxides during storage might be retarded by the presence of antioxidants in rice bran.

5.3.6. Phytochemical composition

Total tocochromanol and oryzanol levels of oil bodies are shown in Figure 5.28. The levels of tocochromanols decreased while those of oryzanol were relatively stable during storage. After being in storage for 40 days at 25 ºC, 41 % of the total tocochromanols had decomposed. The presence of these natural antioxidants in oil bodies may slow down the decomposition of hydroperoxides and reduce the rate of lipid oxidation. Tocochromanols and oryzanol have been reported to scavenge free radicals and prevent formation of hydroperoxides in methyl linoleate bulk and emulsion systems (Nyström, 2007). Furthermore, sitostanyl ferulate (a kind of steryl ferulate in wheat and rye bran comparable to rice bran oryzanol) degraded at a lower rate than α-tocopherol at high temperature (heating at 180 ºC for 6 hours) (Nyström *et al.*, 2007).

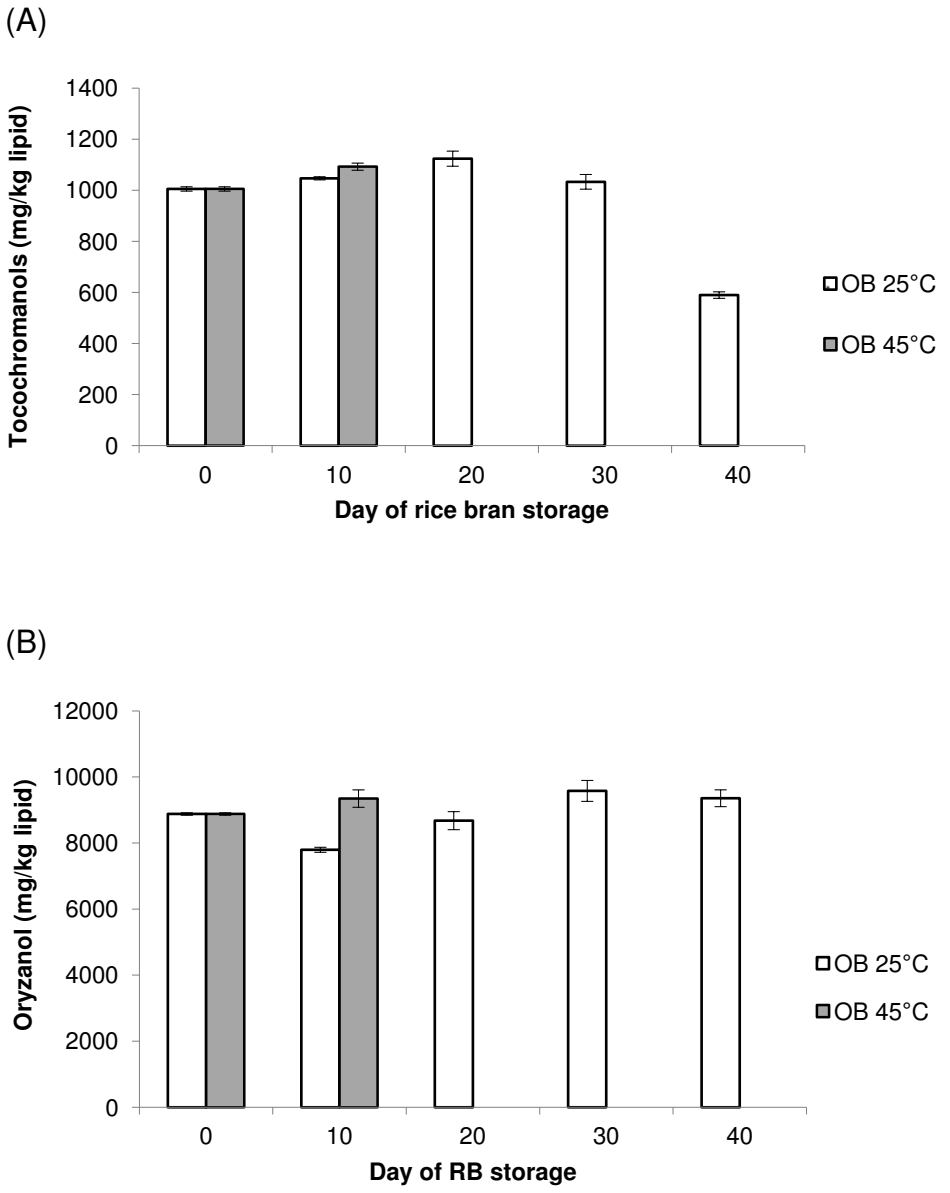


Figure 5.28 Phytochemical concentrations of oil bodies recovered from rice bran stored at 25 ºC and 45 ºC and 75 % relative humidity for 40 days (A) total tocochromanols and (B) oryzanol

Total phenolic content of oil bodies changed slightly and was not consistent during storage of rice bran (Figure 5.29). The small increase of phytochemical concentrations (tocochromanols, oryzanol and total phenolic compounds) during storage may be related to the induction of physiological responses against stressful conditions of rice bran during storage. This leads to accumulation of plant metabolites and antioxidants as discussed previously (section 5.1.3).

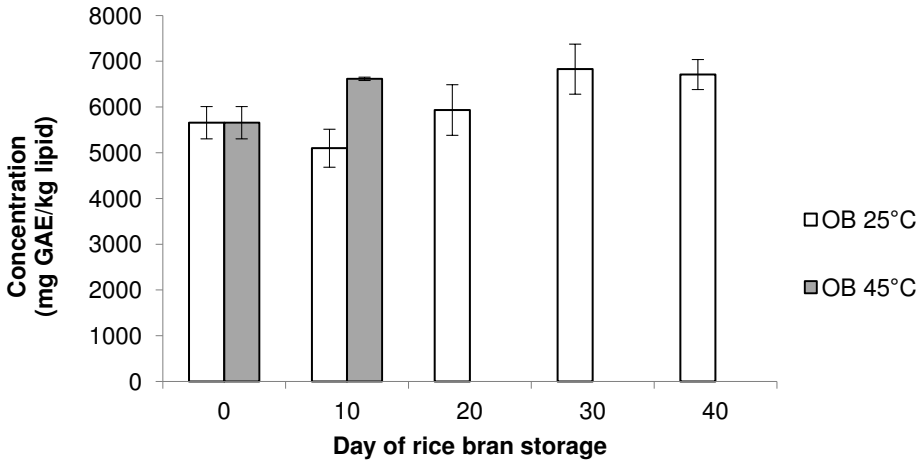


Figure 5.29 Total phenolic content of oil bodies recovered from rice bran stored at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

5.3.7. Antioxidant capacity

Figure 5.30 shows the antioxidant capacity of oil bodies recovered from the stored rice bran. Despite the slight increase of tocochromanol and oryzanol concentrations, the antioxidant capacity of oil bodies decreased after storage.

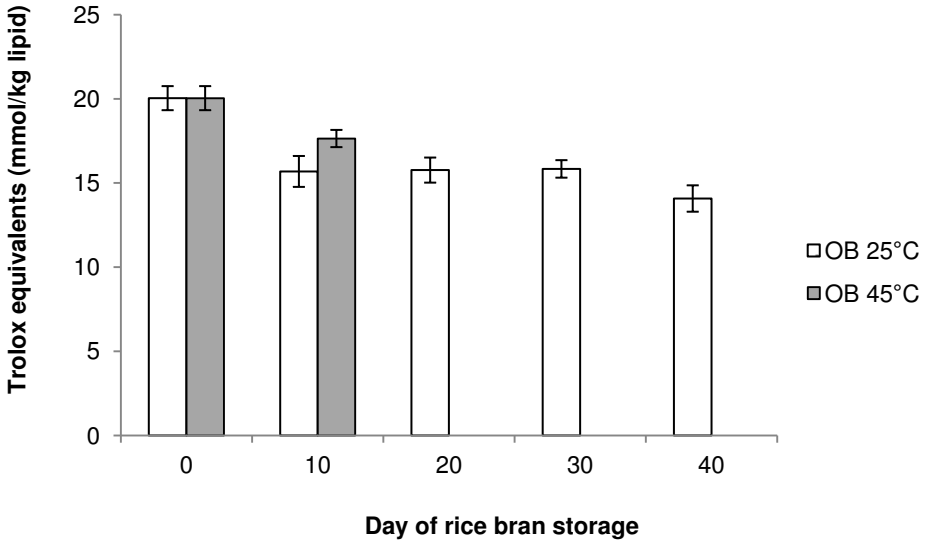


Figure 5.30 Antioxidant capacity of oil bodies recovered from rice bran stored at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

5.3.8. Free fatty acid formation

The major lipid class of oil bodies from freshly milled rice bran is triacylglycerols (lane 1, Figure 5.31). The changes of each lipid fraction of oil bodies followed a similar trend to the parental rice bran during storage. The amount of triacylglycerols in oil bodies decreased while that of free fatty acids increased. In addition, the triacylglycerol mobilization was observed concurrently with the gradual disappearance of oleosins from day 20 (Figure 5.26). This indicated that the main components of oil bodies (triacylglycerol core and oleosin) were damaged during the storage of rice bran.

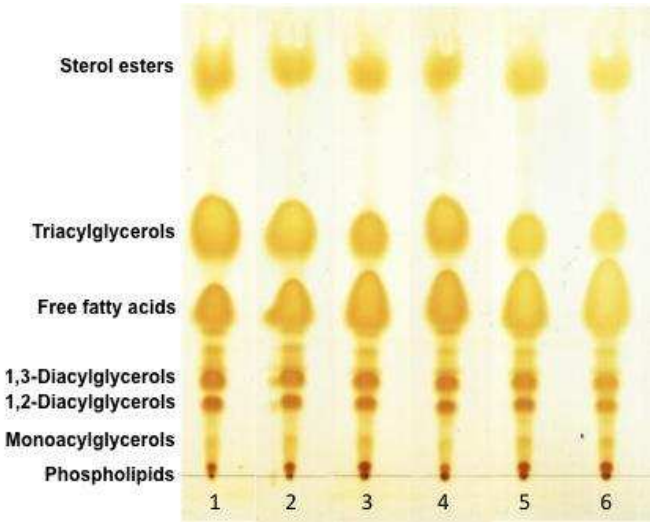


Figure 5.31 Separation of lipid classes of oil bodies recovered from stored rice bran on TLC plate (Silica Gel 60)

Developing solvent: hexane-diethyl ether-acetic acid (80:20:2 by volume). 1, fresh rice bran; 2, rice bran stored 10 days at 25 ºC; 3, 10 days at 45 ºC; 4, 20 days at 25 ºC; 5, 30 days at 25 ºC; and 6, 40 days at 25 ºC

The high FFA levels in oil bodies determined by spectrophotometric method (Figure 5.32) also reflects the high intensity of staining in the region of TLC plate corresponding to FFAs (Figure 5.31). Levels of FFA increased from 17 % in fresh rice bran oil bodies to 64 % in oil bodies recovered from stored rice bran on day 30 at 25 ºC. Since, intact oil bodies have been reported to be relatively resistant towards lipase action (Takano, 1993), oil bodies recovered from fresh bran (assuming that they are intact) are therefore likely to stable against hydrolysis. In contrast, oil bodies recovered from the stored rice bran revealed a carry-over of FFAs that increased with temperature and time of storage. Lipases may access the reserved triacylglycerols substrates through the

deteriorated oleosin surface and then hydrolyze the lipid content of oil bodies to free fatty acids rapidly after day 20.

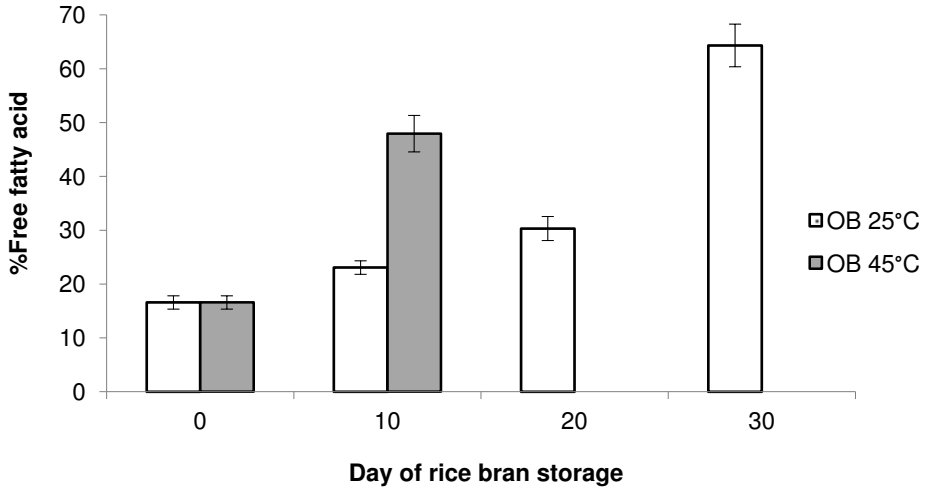


Figure 5.32 Free fatty acid content of oil bodies recovered from rice bran stored at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

Results were calculated as oleic acid and expressed as a percentage (% w/w) of the total lipid content in rice bran

5.3.9. Primary oxidation

Although, levels of polyunsaturated fatty acid (linolenic acid) in oil bodies did not change significantly, lipid hydroperoxides were produced during rice bran storage. Concentrations of the lipid hydroperoxides increased gradually and leveled off after 20 days (Figure 5.33). In addition, the formation of lipid hydroperoxides was more pronounced in the oil bodies recovered from rice bran stored at the high temperature (45 ºC) than the low temperature (25 ºC).

During the early period of the rice bran storage until day 10, the lipid hydroperoxide formation in oil bodies might be retarded by the presence of oleosin (Figure 5.26) at lipid-water interface. The presence of oleosin may retard the lipid oxidation by acting as a physical barrier against pro-oxidants or chelating metals by preventing them from gaining access to the unsaturated fatty acids inside the oil bodies. Presence of antioxidants in oil bodies also contributes to the low lipid oxidation during storage.

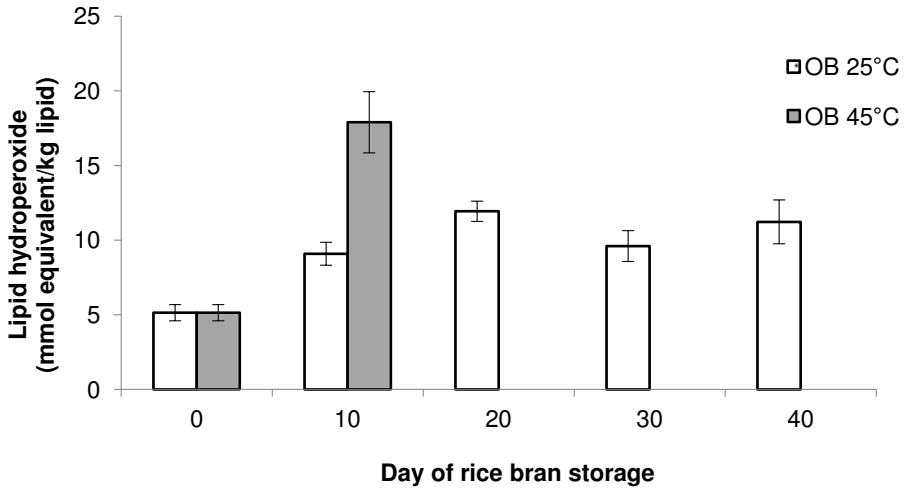


Figure 5.33 Lipid hydroperoxide concentrations of oil bodies recovered from rice bran stored at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

Results were expressed as mmol of cumene hydroperoxide equivalent per kg total lipid content in rice bran

5.3.10. Secondary oxidation

Figure 5.34 shows the change of hexanal level in the oil bodies. No increase of the hexanal level could be observed in any oil bodies recovered from rice bran during storage. This showed that the lipid hydroperoxide decomposition was minimal or the lipid hydroperoxides were broke down to other compounds.

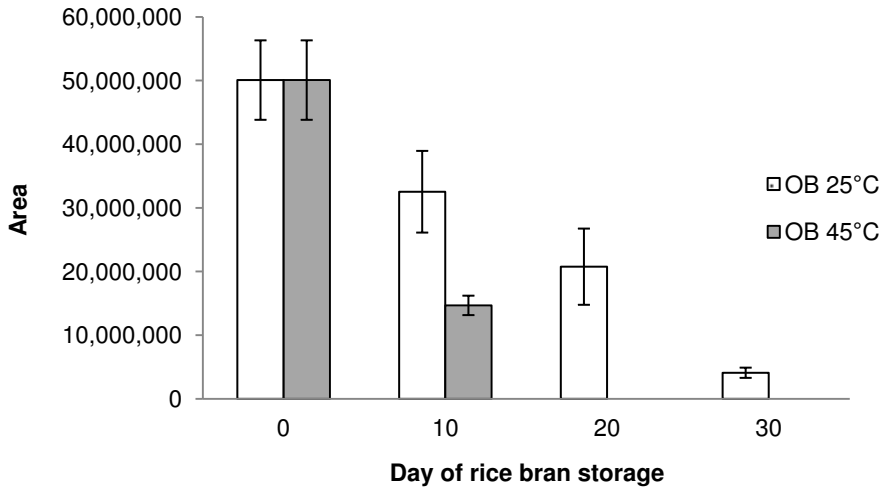


Figure 5.34 The change of hexanal in oil bodies recovered from rice bran stored at 25 ºC and 45 ºC and 75 % relative humidity for 40 days

The oil bodies recovered from freshly milled rice bran did contain a relatively high level of hexanal from the origin (day 0). This may be due to the endogenous hexanal carried over from the aged brown rice (more than 1 year old). However, the level of hexanal was found to be low in fresh rice bran (Figure 5.23). Since, oil body suspension is liquid, the equilibrium of analyte (hexanal) between the

aqueous phase to headspace vapor phase above the sample is achieved easily and rapidly. In contrast, the volatile analysis of rice bran is quite difficult due to the chemisorption of the analyte in the solid matrix (Zhang and Pawliszyn, 1993). Although agitation and heating the sample at 60 ºC was used to increase convection and mass transfer of the analyte, lower level of hexanal was detected in rice bran as compared with the oil body suspension on day 0. In addition, the enrichment of endogenous hexanal and other volatile compounds in the lipid-protein particles that resembled oil bodies have been reported previously (Hudak and Thompson, 1997).

Other secondary oxidation products (thiobarbituric reactive substances, TBARS) produced from the autoxidation of lipids were also measured. However, the formation of TBARS in the oil bodies was too low to be detected correctly (data not shown). It is likely that the lipid hydroperoxides from the primary oxidation do not decompose or decompose to other compounds rather than hexanal and TBARS.

5.3.11. Zeta-potential

The zeta potentials of the oil body suspensions slightly increased and this was accompanied by a small decrease in the pH of the diluted oil body suspension recovered during storage (Table 5.3). This may be attributed to the change of oil body surface

conformation. The oleosins that shield phospholipids were diminished during storage, leaving behind phospholipids (if they still remained) on the surface of the oil bodies. Free fatty acids forming from lipid hydrolysis may be entrapped and physically shielded the surface. The negatively charged phospholipids (phosphatidylserine and phosphatidylinositol) and free fatty acids may contribute to the additional negative charges, electrophoretic mobility and the zeta potential of the oil bodies.

Table 5.3 Zeta potential of oil body suspension from rice bran stored at 25 ºC and 45 ºC and 75 % relative humidity for 40 day [1]

Day of storage	Temperature (℃)	pH of diluted suspension	Zeta potential (mV)
0	-	6.8 ± 0.0	31.2 ± 2.3
10	25	6.7 ± 0.1	36.3 ± 2.8
	45	6.7 ± 0.0	33.6 ± 0.6
20	25	6.7 ± 0.0	33.1 ± 0.9
	45	ND	ND
30	25	6.6 ± 0.0	37.6 ± 0.3
	45	ND	ND
40	25	6.4 ± 0.0	35.6 ± 0.8
	45	ND	ND

[1] ND = not determined

To summarize, the main component of rice bran lipids is triacylglycerols, which are stored in small organelles known as oil bodies. In this study, the presence of oil bodies in the recovered oil body material was confirmed by the light micrographs, the small particle size distribution, and the appearance of oleosin candidate bands on the SDS-PAGE. It can be seen that the morphology and

characteristics of the oil bodies were changed and weakened during the storage of parental rice bran that resulted in the release of lipids from their structures. Particle size of the recovered oil body increased. There was a relative decrease in oleosin from day 20 and day 10 of the storage trial at 25 ºC and 45 ºC respectively. When the oil bodies started to disintegrate, the inside lipid and surface protein of the oil bodies were exposed to cytoplasm and then deteriorated by hydrolysis and oxidation. As a result, the total lipid content, oil body lipid recovery yield and relative triacylglycerol intensity on TLC decreased, while the FFA levels significantly increased. However, the oil bodies that could be recovered from the stored rice bran still contained high levels of phytonutrients that would be useful for rice bran oil extraction commercially.

5.4. Rice bran stabilization

Prevention of lipid degradation is necessary in order to improve yield and quality of recovered oil bodies. Rice bran stabilization not only prevents the lipid breakdown, but also helps to control growth of microorganisms and insects. Classical methods of rice bran stabilization include dry heat treatment, wet heat treatment, extrusion, refrigeration, pH modification and chemical treatment (Orthoefer, 2001). Heat treatment at atmospheric pressure was selected in this study because it was simple and simultaneously inactivated lipases by lowering water required for the hydrolysis.

There are two methods of rice bran stabilization in this section; hot air stabilization and extrusion stabilization.

5.4.1. Hot air stabilization

The hot air stabilization or pan roasting is a dry heat treatment process. Freshly milled rice bran (derived from brown rice aged more than one year old) was heated on a tray in a hot air oven at 110 ºC for 0, 1, 5, 10 and 20 min (see section 2.2.1). Despite the simplicity of this method, the heating may not be uniform and the satisfactory level of stabilization may not be achieved. The long heating time resulted in dark colour of the heat-treated bran and its lipid (data not shown). Above all, the hot air drying caused oil body fusion and coalescence (Figure 5.35).

The hot air drying statistically reduced ($P<0.05$) the rice bran moisture content with the increasing heating time (Table 5.4). Heating rice bran for 1 to 5 minutes resulted in an increase of total lipid content. This may be due to the increase of lipid extractability and the release of contaminated non-polar compounds from heat-treated rice bran to the isooctane lipid extracts.

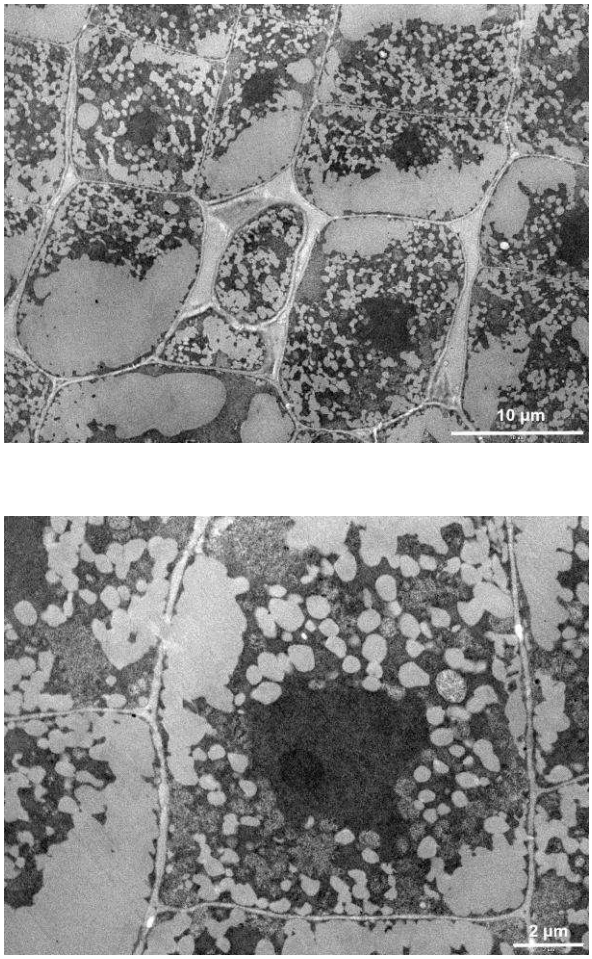


Figure 5.35 Transmission electron micrographs of hot air stabilized fresh rice bran at 110 ºC for 20 minutes

Table 5.4 Chemical composition of stabilized rice bran (RB)

Sample	%MC	Total lipid (% dwb)	Protein (% dwb)
Fresh RB	6.6 ± 0.0	20.7 ± 0.6	20.9 ± 1.2
Extruded RB	3.7 ± 0.2	20.1 ± 0.8	8.0 ± 0.4
RB heat 1 min	5.6 ± 0.0	25.4 ± 1.7	14.5 ± 2.7
RB heat 5 min	2.4 ± 0.1	22.5 ± 0.7	17.1 ± 1.2
RB heat 10 min	0.6 ± 0.1	20.2 ± 0.7	17.9 ± 2.3
RB heat 15 min	0.6 ± 0.1	20.8 ± 0.8	17.8 ± 1.0
RB heat 20 min	0.2 ± 0.1	21.2 ± 0.3	18.2 ± 0.5

TLC plate suggested that the freshly milled rice bran in this experiment was rich in FFA even only within a few hours after milling, but no increase in the FFA of the total lipid extracts from heat-treated rice bran (Figure 5.36) and the lipids from the recovered oil bodies (Figure 5.37).

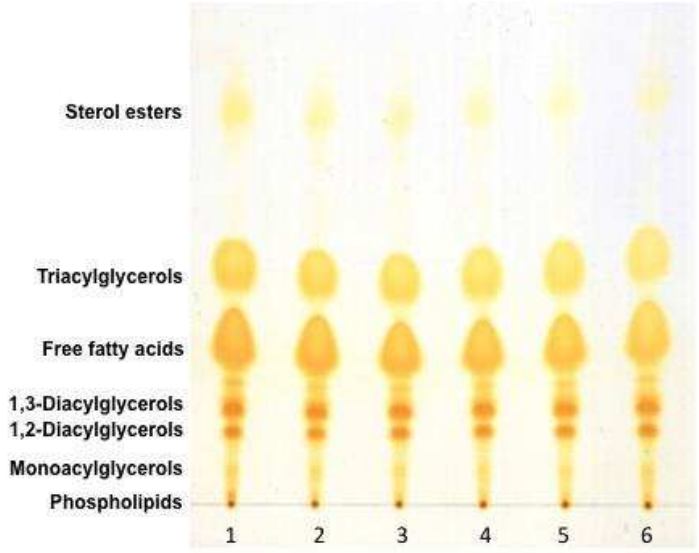


Figure 5.36 Separation of lipid classes of heat-treated rice bran lipids on TLC plate (Silica Gel 60)

Developing solvent: hexane-diethyl ether-acetic acid (80:20:2 by volume). 1, extruded rice bran lipids; 2, heat-treated rice bran at 110 ºC for 1 minute; 3, 5 minutes; 4, 10 minutes; 5, 15 minutes; and 6, 20 minutes

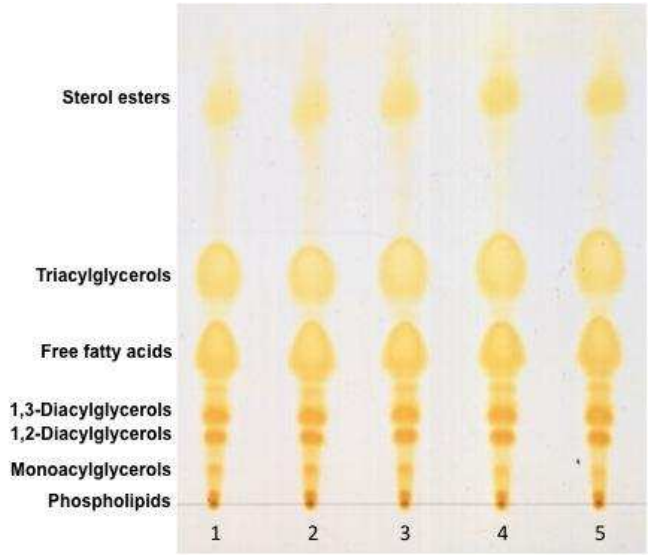


Figure 5.37 Separation of lipid classes of oil bodies recovered from heat-treated rice bran on TLC plate (Silica Gel 60)

Developing solvent: hexane-diethyl ether-acetic acid (80:20:2 by volume). 1, oil bodies recovered from heat-treated rice bran at 110 ºC for 1 minute; 2, 5 minutes; 3, 10 minutes; 4, 15 minutes; and 5, 20 minutes

Tocochromanol and oryzanol concentrations of rice bran also statistically decreased ($P<0.05$) with the increase in heating time (Table 5.5). However, oryzanol was relatively more stable to the heat treatment than tocochromanols. Stabilized rice bran at 110 ºC for 20 minutes lost 28 % and 17 % of its tocochromanols and oryzanol respectively. The fatty acid composition of the heat-treated rice bran was not changed as compared with the non-heat-treated raw rice bran (Figure 5.38).

Table 5.5 Phytochemical concentration of stabilized rice bran [1]

Sample	Total tocochromanol (mg/kg lipid)		Oryzanol (mg/kg lipid)	
	Rice bran	Oil bodies	Rice bran	Oil bodies
Fresh RB	1,170 ± 10	992 ± 15	11,213 ± 163	8,193 ± 306
Extruded RB	1,066 ± 12	ND	10,870 ± 65	ND
RB heat 1 min	1,024 ± 4	964 ± 9	11,689 ± 14	7,530 ± 52
RB heat 5 min	968 ± 4	953 ± 11	11,223 ± 94	7,076 ± 120
RB heat 10 min	938 ± 4	885 ± 7	10,976 ± 19	6,761 ± 77
RB heat 15 min	939 ± 5	891 ± 13	10,168 ± 31	6,973 ± 180
RB heat 20 min	837 ± 7	886 ± 12	9,306 ± 138	6,764 ± 61

[1] ND = not determined

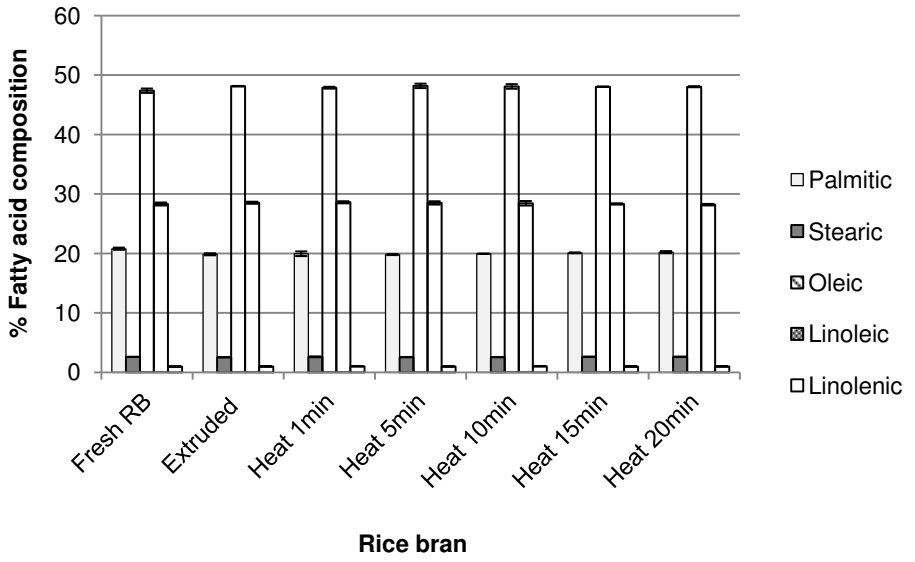


Figure 5.38 Fatty acid composition of stabilized rice bran

There was a statistical ($P<0.05$) decrease of phytochemical concentrations, total lipid content and oil body lipid recovery of the oil bodies recovered from rice bran after hot air stabilization (Table 5.6). However, the fatty acid composition of the recovered oil bodies from heat-treated rice bran did not change (Figure 5.39).

Table 5.6 Chemical composition of oil bodies recovered from stabilized rice bran [1]

Sample	Total lipid (%dwb)	Protein (%dwb)	% OB lipid recovery
Fresh RB OB	84.7 ± 3.0	9.9 ± 0.3	24.2 ± 2.3
Extruded OB	ND	ND	ND
OB heat 1min	84.6 ± 0.7	8.2 ± 0.3	18.9 ± 1.5
OB heat 5 min	81.2 ± 1.3	9.5 ± 0.5	19.7 ± 1.1
OB heat 10 min	80.3 ± 1.0	10.1 ± 0.6	19.6 ± 0.3
OB heat 15 min	76.0 ± 1.7	9.8 ± 0.2	16.6 ± 2.1
OB heat 20 min	78.2 ± 0.9	8.8 ± 0.3	14.5 ± 2.1

[1] ND = not determined as there was only trace amount of oil bodies from extruded rice bran

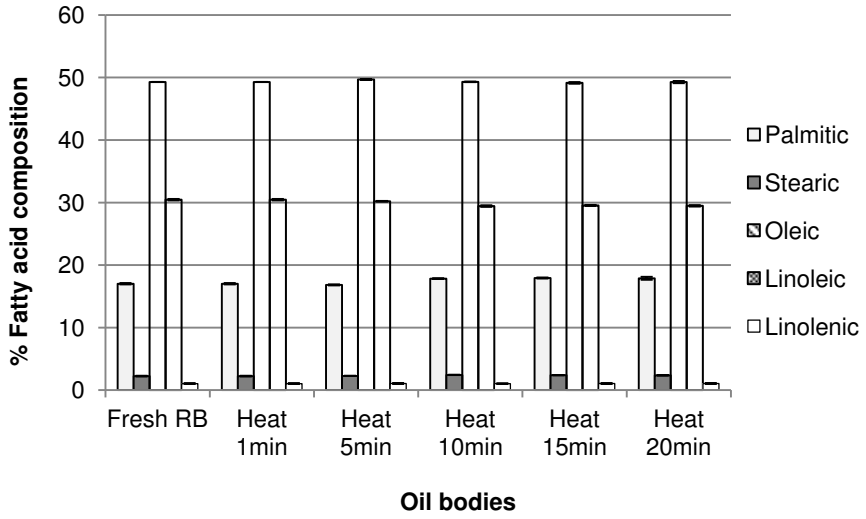


Figure 5.39 Fatty acid composition of oil bodies recovered from hot air stabilized rice bran

Oil bodies recovered from the hot air stabilized rice bran were larger in diameter than that of the non-heat-treated raw rice bran, with the maximum diameter of 10 μm (Figure 5.40). The relative turbidity and rate of creaming of the oil body suspension are extremely low in the oil bodies recovered from rice bran treated for

more than 10 minutes (Figure 5.41 and Figure 5.42). This shows that the stability of oil body suspension decreased with the increase of rice bran heating time, especially at long duration of more than 10 minutes at 110 ºC.

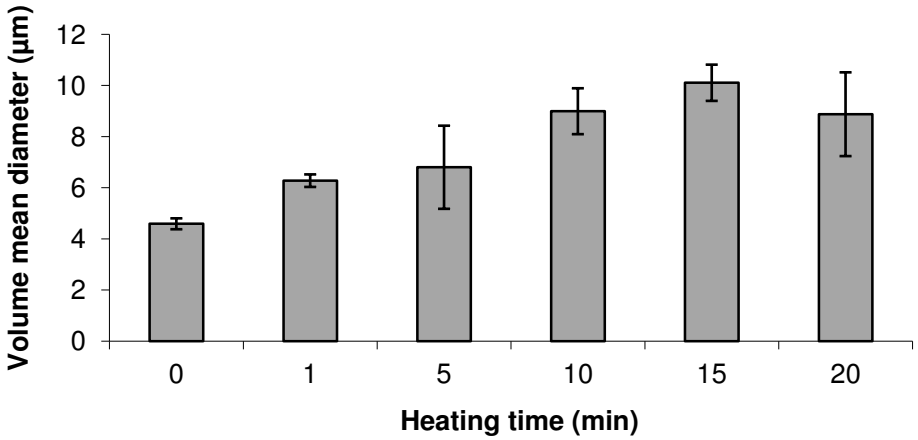


Figure 5.40 Particle size of oil bodies recovered from hot air stabilized rice bran

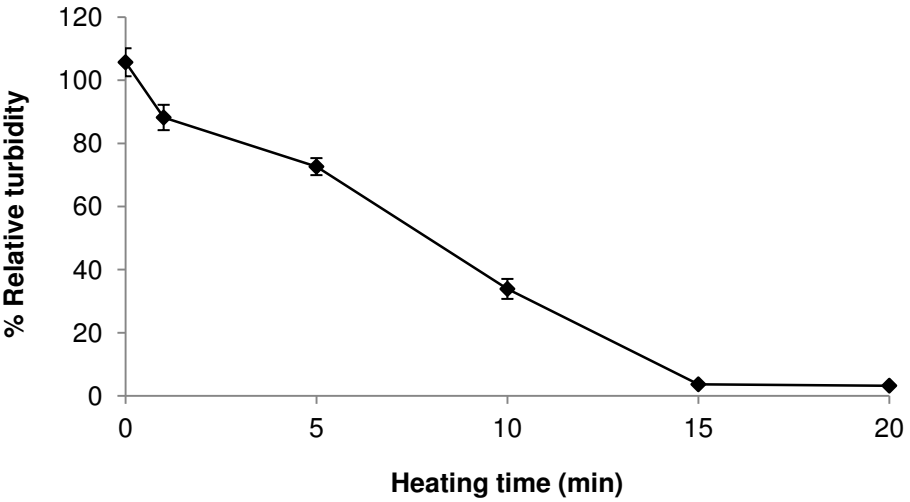


Figure 5.41 Relative turbidity after 24 hours of oil body suspension prepared from hot air stabilized rice bran (pH 7.0 ± 0.1)

Figure 5.42 Stability test after 24 hours of oil body suspension prepared from hot air stabilized rice bran

Oil body suspension prepared from heat-treated rice bran, heated for A, 1 min; B, 5 min; C, 10 min; D, 15 min; and E, 20 min

To conclude, hot air drying offers the simplicity of stabilization process at a low cost. However, uniform heating may not be achieved. The long heating time at a high temperature reduced the phytochemical concentrations of rice bran and oil bodies. Heating rice bran at 110 ºC for more than 10 minutes caused the coalescence of oil bodies and the instability of the oil body suspension.

5.4.2. Extrusion stabilization

Extrusion stabilization is used commercially. The time of the process is very short in order to avoid any burning that will give a dark colour and loss of phytonutrients in the stabilized rice bran. FFA levels of the stabilized rice bran (3 min hold at 130 ºC) was reported

to increase only slightly (1 - 2 %) over a 135 day storage period at 32 ºC (Randall *et al*., 1985).

In this study, rice bran was extruded as described in section 2.2.2. The extrusion cooking uses retained moisture heating method. The moisture was retained under pressure (5 Bar) until completion of the heating phase. Rice bran (2kg/hr) was pre-heated at 80 ºC before heating it up to 130 ºC for 3 minutes by friction and mechanical energy at an outlet where water flashed to steam (screw speed 200 rpm and torque per shaft 10 Nm). The extruded rice bran exited the extruder as porous pellets and was darker in colour as compared with the raw rice bran (Figure 5.43). Rice bran is commercially extruded into pellets to increase solvent penetration during oil extraction and to facilitate removal of fine bran residue from refined oil (Sayre *et al.*, 1982).

Like the hot air stabilized rice bran, the oil bodies recovered from extruded rice bran were coalesced and large in size (Figure 5.44). In addition, the oil bodies were extremely damaged as can be seen by the loss of surface integrity and fractures all over the oil body droplets.

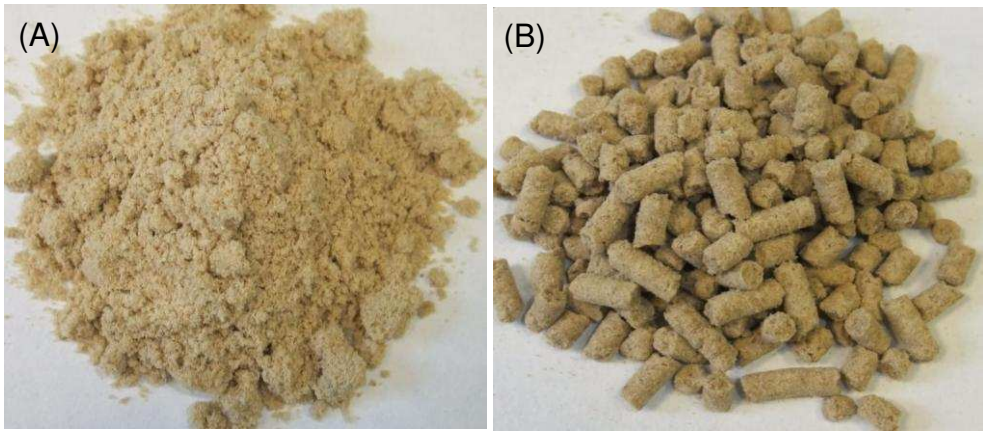


Figure 5.43 Images of (A) raw rice bran and (B) extruded rice bran

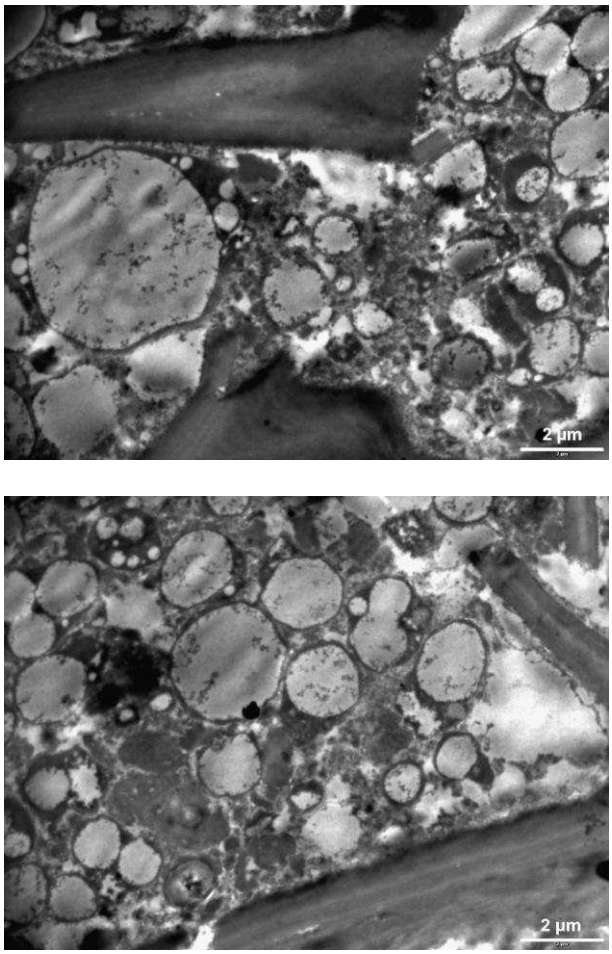


Figure 5.44 Transmission electron micrographs of extruded rice bran

The moisture content of the extruded rice bran decreased to 3.7 % (Table 5.4). Tocochromanol and oryzanol concentrations of the extruded rice bran significantly decreased ($P<0.05$) after the extrusion (Table 5.5). Oryzanol was relatively more stable during the extrusion process than tocochromanols. Extruded rice bran lost 9 % and 3 % of its tocochromanols and oryzanol respectively. This indicated that the decomposition of rice bran phytochemicals in the hot air drying was higher than that of the extrusion stabilization. The extrusion caused an increase in particle size and bulk density of the extruded rice bran because of the agglomeration into large pellets (Table 5.7).

Table 5.7 Particle size and bulk density of extruded rice bran

Mesh	Particle size (μm)	%Particle size distribution	
		Raw rice bran	Extruded rice bran
12	> 1,700	0.2 ± 0.0	88.5 ± 2.5
22	699-1,700	0.5 ± 0.0	2.2 ± 0.9
30	600-699	1.5 ± 1.8	0.7 ± 0.1
40	425-600	30.3 ± 1.2	1.2 ± 0.6
45	355-425	41.6 ± 1.2	2.1 ± 0.8
< 45	< 355	25.1 ± 3.4	4.9 ± 0.7
Bulk density (kg/L)		0.32 ± 0.01	0.49 ± 0.01

Disappointingly, an attempt to recover oil bodies from the extruded rice bran was unsuccessful. This was due to the fact that the extrusion promoted instability/disintegration of the oil body structure, or created a matrix effect in the bran, which limited the oil body recovery. Additionally, protein content of the extruded rice bran

was significantly decreased ($P<0.05$) (Table 5.4) indicating that the process may cause oleosin and protein degradation. The extrusion stabilization may improve the speed and the extent of oil extraction but not the oil body recovery. The recovered lipid material was much larger in diameter (10 - 20 μm) as compared with the untreated oil bodies and stained in pink by Oil Red O (Figure 5.45). This indicated that the recovered material was free oil and not the oil bodies.

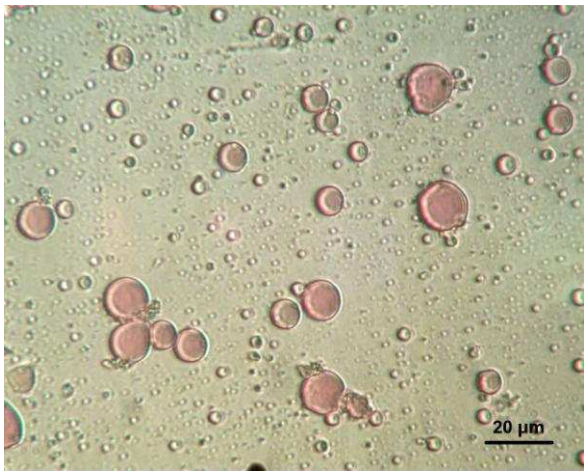


Figure 5.45 Light micrograph of recovered lipids from extruded rice bran.

Free oil was stained in pink by Oil Red O

Other stabilization methods that have been used to inactive enzymes including refrigeration, pH modification, chemical treatment, and microwave heating. However, storing rice bran at low temperature is costly. Addition of acids or other chemicals may not be uniform and affect consumer acceptability. Therefore, these

methods are not currently practiced on commercial applications. In addition, stability of oil bodies recovered from the chemically-treated bran may be reduced as they are highly influenced by the pH and charged particles in suspension. Microwave heating might be an option for stabilizing rice bran, however, integrity of recovered oil bodies from the microwaved rice bran remains unknown.

5.5. Conclusions

Both moisture and temperature in rice bran have an impact on the lipid degradation during storage. Increasing temperature and relative humidity during the rice bran storage increased the rate of lipid hydrolysis (hydrolysis of TAGs to FFAs) and oxidative rancidity (changes of lipid hydroperoxide).

Tocotrienols, tocopherols, oryzanol and several phenolic compounds exhibit beneficial effects such as antioxidant activity against lipid deterioration during storage. The destruction of tocochromanols was related to the lipid oxidation and depended not only on the temperature, but also the moisture content, humidity, thermal stabilization and microbial growth during storage. The tocochromanol concentrations of rice bran decreased in a temperature dependent fashion while those of oryzanols were relatively steady suggesting that oryzanol was more stable than tocochromanols in the storage conditions investigated. Despite the

high concentration of antioxidants, raw rice bran was highly oxidized during storage.

The degradation of both protein and lipid in rice bran during storage could have negative effects on the recovery and the quality of recovered oil bodies. Naturally, oil bodies are covered by a layer of phospholipids and oleosin proteins, which provides physical and chemical protection for long term storage of natural lipids against environmental stresses and before seed germination. The storage of rice bran resulted in the decrease of oil body lipid recovered yields, reduction of oleosin and loss of oil body integrity and stability. This indicated that oil bodies were decomposed during the hydrolysis of rice bran lipids. The oxidation increased rapidly after oil bodies disintegrated. In addition, the oil bodies that could be recovered from the stored rice bran still contained high levels of tocochromanols and oryzanol indicating that the preexisting natural protection of the oil bodies could preserve the phytonutrients in rice bran during storage and at a high temperature.

The oil bodies can delay the onset of lipid oxidation of stored lipids inside the oil bodies by the intrinsic association between the oil bodies and phytochemicals in rice bran and the presence of oleosin. The oleosin of the intact oil bodies may slow down the lipid hydrolysis by acting as a physical barrier to lipid degrading enzymes. Therefore,

oil bodies may prove to be a novel approach based on nontoxic, nonvolatile solvent that may simultaneously extract oil and natural antioxidants from rice bran that can be applied for commercial practice in food industries.

Due to the rapid degradation of lipid in rice bran immediately after milling and during storage, stabilization should be done to prevent the lipid breakdown. This is most easily achieved by heating to inactivate all enzyme activities. In the present study, the hot air stabilization and extrusion stabilization were performed. The moisture content and phytochemical concentrations of the stabilized rice bran decreased significantly ($P<0.05$). The high temperature of stabilization caused oil body fusion and coalescence, which limit the recovery of the oil bodies from the stabilized rice bran. Therefore, the fresh, non-heat-treated rice bran is preferred for the recovery of intact oil bodies from rice bran.

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1. General discussion

This work could be used to indicate a potential of recovery of oil bodies from rice bran, a by-product from rice milling. The recovery of rice bran oil bodies would be useful in recommending an innovative way of processing rice bran for human consumption. Although, this data was generated in a laboratory scale, it should be possible to scale up for mass production. The commercial production of rice bran oil bodies will increase the value of rice bran and rice bran oil in the market. Enrichment of phytochemicals in rice bran oil bodies features them as an innovative product for niche market. Oil bodies may act as delivery vehicles for antioxidants, especially in emulsion systems. Tocochromanols and oryzanol in rice lipids are well known for hypocholesterolemic activity, serum cholesterol reduction, increasing neutral sterols and bile acid excretion and anticancer properties (Champagne, 2004).

Worldwide production of rice paddy and rice bran oil were 523 and 0.83 million tonnes respectively in 2007 (FAOSTAT, 2007). Based on the assumption of 10 % bran from paddy and approximately 15 % oil extraction from bran, only 11 % of rice bran from milling was used for rice bran oil production. Rice bran is

normally used as a feed ingredient due to its protein and lipid content. To the food industry, rice bran is traded only in limited quantities partially due to its instability.

Extraction of rice bran oil is particularly challenged by availability of quality rice bran. Rice bran oil is typically used as flying oil because of its pleasant flavour over other oils and oxidative stability comparable to that of peanut and cottonseed oil, partly due to its low linolenic acid content (Orthoefer, 1996). Rice bran oil is also used as an ingredient in food products such as confectionery, salad dressing, mayonnaise, margarines and shortening, and non food products including feed, soaps, glycerin, cosmetic and polish products (Champagne, 2004).

Lipids in cereals are generally distinguished into starch lipids and non-starch lipids (Morrison, 1988). The starch lipids are lipids inside native starch granules of endosperm and are composed exclusively of monoacyl lipids. The non-starch lipids are the major lipids present in rice consisting of membrane and spherosome lipids (Champagne, 2004). Spherosomes (i.e. oil bodies) are present in the aleurone layer, germ and subaleurone layer of starchy endosperm (Juliano, 1983). They are composed mainly of triacylglycerols, diacylglycerols and phospholipids, but FFAs and other monoacyl lipids are also present and are caused by lipid

hydrolysis during storage (Morrison, 1988). The location of lipids and their composition in rice bran are consistent with our observations in this study.

The bran fraction is significantly enriched in lipid, protein, tocochromanols and oryzanol as compared with brown rice and white rice. The recovery of oil bodies from whole grain brown rice and white rice were limited by the presence of starch and proved to be too inefficient for commercial application. The focus of this investigation was therefore on oil bodies recovered from the bran only. This study shows that oil bodies can be recovered and purified from rice bran. Rice bran oil bodies were recovered by wet milling with water or an alkali-solution, filtration, centrifugation and collection of creamy crude oil body pad. Mechanical forces applied during the wet milling help breaking up rice bran cell wall and release of oil bodies into the extraction medium. The recovered crude oil body pad was relatively stable (i.e. no evidence of free oil layer or rancid smell) up to five days after the recovery. The crude oil bodies were further washed to remove residual cell debris and passively associated proteins with different washing media including water, urea and alkali solution.

The processes of oil body recovery and washing could be more environmentally friendly compared with the conventional solvent extraction of rice bran oil using toxic and explosive solvents such as hexane. The oil body material also has a potential as a functional food ingredient since the recovery is easy, and emulsification is achieved by simply dispersing the oil bodies in their natural form in an aqueous medium.

In this study, size of oil droplets in the recovered oil body fraction was similar to rice bran oil bodies *in vivo*. Presence of oleosins in the recovered oil body fraction by SDS-PAGE also confirmed the retention of oil bodies in our recovered lipid material. However, one needs to be cautious about assuming that even this described oil body material is composed of intact oil bodies. Integrity of rice bran oil bodies can be indirectly measured by the degree of oil body destabilization (large and duplex droplets and/or coalescence leading to the release of free oil). When the oil bodies were processed with urea, significant damage to oil bodies occurred. In the presence of 0.1 M sodium bicarbonate, either softened the cell wall during milling and to retained small oil droplets, or the alkaline pH restricts the size of hybrid oil droplets, and perhaps the extent of coalescence, by inducing a net negative charge on the surface of the oil bodies, so promoting repulsion rather than aggregation of neighboring oil bodies.

The fatty acid composition of total lipids in rice bran, crude and washed oil bodies was similar (Table 6.1). However, on a relative % basis, alkali-COB contains less saturated and higher unsaturated fatty acids than rice bran. The difference of fatty acid composition between rice bran and oil bodies might be an artifact of oil body recovery; in another words, some of the free saturated fatty acids were left behind and separated from oil bodies because they bound with protein or phospholipids in rice bran, other free saturated fatty acids may be forced to associate with protein such as oleosin during drying of oil body material (dehydrated form of oil bodies) before solvent extraction of total lipids or may be bound to phospholipids on the oil body surface and thus were not recovered with non-polar solvent (isooctane). The latter hypothesis could be tested by recovering total lipids from a wet oil body preparation using the Bligh and Dyer method.

Table 6.1 Comparison of fatty acid composition of total lipids in rice bran and oil bodies

Fatty acid	% Composition		
	Rice bran	Alkali-COB	Alkali-WOB
Palmitic acid	20.8	17.2	17.4
Stearic acid	2.6	1.3	1.0
Oleic acid	47.4	53.1	53.9
Linoleic acid	28.3	27.8	27.3
Linolenic acid	1.0	0.6	0.4
Saturated fatty acids	23.4	18.4	18.4
Unsaturated fatty acids	76.6	81.6	81.6

Enzyme-assisted recovery was first aimed to recover intact oil bodies and may therefore provide the ideal material to test the hypothesis that the change of oil body stability and size during washing are due to the presence of hybrid oil droplets. However, oil bodies released from the bran through enzymes were large and low in quantity. Total sugar release data suggested that the cellulase was not active at the selected conditions or the incubation (2 hours) was not long enough for the enzyme to complete reaction. Since, high temperature of the incubation (50 ºC) was set to activate cell wall degrading enzyme, other endogenous enzymes in rice bran were also activated. This limits the use of enzymes for rice bran oil body recovery. In contrast, commercial mixtures of cell wall degrading enzymes incubated at much longer time (20 hours) have been previously reported to effectively isolate oil bodies with lots of membrane debris from dry soy bean flour (Kapchie *et al.*, 2010). Long incubation times cannot be applied to rice bran since an attempt to soak rice bran (24 hours) at room temperature resulted in lipid hydrolysis and the loss of oil bodies. Stabilization of rice bran by microwaving to inactivate any endogenous enzymes prior to the incubation, may be useful. The enzyme-assisted recovery of oil bodies from microwaved rice bran may then be achieved.

To the best of our knowledge, this is the first study providing information on characteristics and phytochemical content of rice bran oil bodies *ex vivo*. Moreover, this study is the first to demonstrate an association between the phytonutrient tocochromanols & oryzanol, and oil bodies in rice bran. Tocochromanols and oryzanol appear to be physically associated with rice bran oil bodies *ex vivo*. They are more strongly associated with oil bodies than general phenolic compounds, some of which are probably passively associated with extraneous proteins, which are removed by washing. Oryzanol is even more strongly associated than tocochromanols. The washed oil body preparation contained approximately 35 - 68 % of tocochromanols and 60 - 62 % of oryzanol present in parent rice bran oil (Table 6.2). This is a remarkable result from rice bran oil bodies and is consistent with previous oil body work on sunflower tocopherol (White *et al.*, 2009), soybean isoflavones and saponins (Kapchie *et al.*, 2011) and oat tocochromanols (White *et al.*, 2006). The phytochemical concentration does decline in the washed preparation from both oil body recovery methods. Perhaps there are extraneous materials that are also enriched with tocochromanols and oryzanol, which are washed away in the process, or the strength of their association is weaker than that of oleosin with oil bodies.

Table 6.2 Recovery yield of tocochromanols and oryzanol in oil bodies relative to parent rice bran oil [1]

Sample [2]	% Recovery	
	Tocochromanols	Oryzanol
Rice bran oil	100	100
COB	46	68
WWOB	46	67
UWOB	35	62
Alkali-COB	70	63
Alkali-WOB	68	60

[1] % Recovery was calculated from the concentration (mg/kg lipid) of tocochromanols or oryzanol in the oil bodies divided by the concentration in rice bran oil x 100 %

[2] COB, crude oil bodies; WWOB, water washed oil bodies; UWOB, urea washed oil bodies; Alkali-COB, alkaline-crude oil bodies; and Alkali-WOB, alkali-washed oil bodies

Although functions of tocochromanols and oryzanol within rice bran oil bodies remain unclear, the existence of tocochromanols was reported previously to protect oil and/or membranes against lipid oxidation *in vivo* (Fryer, 1992). Plant sterols have been reported to play important roles in the regulation of membrane fluidity including storage and transport, and the stability of plant lipid membranes (Piironen *et al.*, 2000). An increase of sterol to phospholipid ratio leads to membrane rigidification (Itzhaki *et al.*, 1990).

The concentrations of tocochromanols and oryzanol in the present study and the literature values are compared in Table 6.3. The crude rice bran oil was commercially produced using a continuous solvent extraction (hexane). Due to the different sources of rice bran for oil extraction, the concentrations of these

phytochemicals in isooctane total lipid extracts (present study - Indian origin) were much lower than those of crude rice bran oil (literature - Thai origin). In this study, tocotrienols, tocopherols and oryzanol represented about 0.10 %, 0.04 % and 1.42 % of rice bran lipids respectively. The oil bodies recovered from rice bran were more enriched in tocochromanols than the rice bran oil extracted by the conventional solvent-based method.

Table 6.3 Comparison of phytochemical concentrations between the present study and the literature values [1]

Source	Sample [2]	Concentration (mg/kg lipid)	
		Tocochromanols	Oryzanol
Present study	Rice bran (methanolic extracts)	1,430	14,174
	Rice bran total lipid extracts (isooctane extracts)	329	7,311
	MP-COB	864	6,995
	COB	656	9,692
	WWOB	658	9,538
	UWOB	499	8,830
	Alkali-COB	1,006	8,880
	Alkali-WOB	974	8,503
Literature	Crude RBO	550	21,100
	Degummed RBO	640	-
	Dewaxed RBO	640	20,000
	Neutralized RBO	530	2,500
	Bleached RBO	240	2,800
	Deodorized RBO	180	2,300

[1] Adapted from Hoed *et al.* (2010)

[2] MP-COB, mortar and pestle oil bodies; COB, crude oil bodies; WWOB, water-washed oil bodies; UWOB, urea-washed oil bodies; Alkali-COB, alkaline-crude oil bodies; Alkali-WOB, alkali-washed oil bodies; and RBO, rice bran oil

Indeed, purified oil bodies also contained higher levels of tocochromanols and oryzanol than the refined rice bran oil. Both tocochromanol and oryzanol concentration in commercial rice bran oil decreased significantly during physical refining. Rice bran oil refining was reported to cause substantial losses of oryzanol especially at the neutralization step (Pestana *et al.*, 2008). Therefore, the oil bodies recovered from rice bran via a simple wet milling process may prove to be an innovative approach based on nontoxic, nonvolatile solvent that may simultaneously extract oil and natural antioxidants including tocochromanols and oryzanol from rice bran. This supports the concept of oil bodies as functional food ingredients.

Lipid degrading enzymes and rice bran lipids are segregated in the intact rice kernel. However, after rice milling, rice bran lipids are exposed to lipases. This results in rapid hydrolysis of triacylglycerols to free fatty acids and glycerols. Lipid oxidation of free fatty acids generates hydroperoxides, which further transform into various secondary products. The lipid hydrolysis and oxidation cause an increase of acidity, unacceptable functional properties and undesirable organoleptic characteristics which limit the use of rice bran as food ingredients and edible oil extraction (Goffman and Bergman, 2003).

In this study, the rate of oxidation of rice bran increased when temperature was increased (45 ºC > 37 ºC > 4 ºC) during storage. However, evaporation of moisture at the high temperature could occur during storage. Therefore, the effect of temperature on the lipid oxidation in a close system (controlled relative humidity) during the storage was studied. Under the controlled humidity (75 %) and high water activity (a_w 0.7), temperature has an effect on the lipid oxidation. Lipid hydroperoxide level increased with an increasing temperature. In this experiment, rice bran seemed to have less antioxidant capacity at the high water activity. The moisture in rice bran may increase mobilization of catalysts (such as enzymes involved in the oxidation and trace metals) to lipid-water interface, thus facilitating the lipid oxidation. In addition, the lipid oxidation is accelerated by pro-oxidants such as lipoxygenases, singlet oxygen, and transition metals especially iron, which might be contaminated in rice bran from rice milling machine.

One drawback of this study is the high level of FFAs in freshly milled rice bran. This is because the freshly milled rice bran refers to the bran mill on site but it is milled from brown rice that is over one year old. The availability of freshly harvested rice paddy is limited in the country (UK). Also, there were occasions when oil body recovery and lipid extraction was carried out 1 - 2 day after milling, so the bran was even less fresh.

A number of studies showed the effects of storage conditions and duration on the stability of rice bran, rice bran oil and rice bran oil in water emulsions (Charoen *et al.*, 2012; Shin *et al.*, 1997; Suzuki *et al.*, 1996; Takano, 1993). However, there was no information available on the effects of the storage of rice bran on its oil bodies. The influence of storage time, temperature and humidity on chemical composition, phytochemical concentration and stability of rice bran oil bodies were investigated in this study. Oil bodies were recoverable from the stored rice bran, but their yield appeared to decline after the rice bran storage for 10 days. Disintegration of oil bodies was observed by the change of oil body droplets (large duplex formation, aggregation and coalescence) and depletion of oleosin covering oil body surface (Figure 6.1). It is likely that the disintegration of oil bodies initiates the decomposition of triacylglycerols. Serious damage at the oil body surface membrane may cause a leakage of triacylglycerols out of the oil bodies. This is followed by decomposition of triacylglycerols causing an increase in free fatty acids and rancidity during storage.

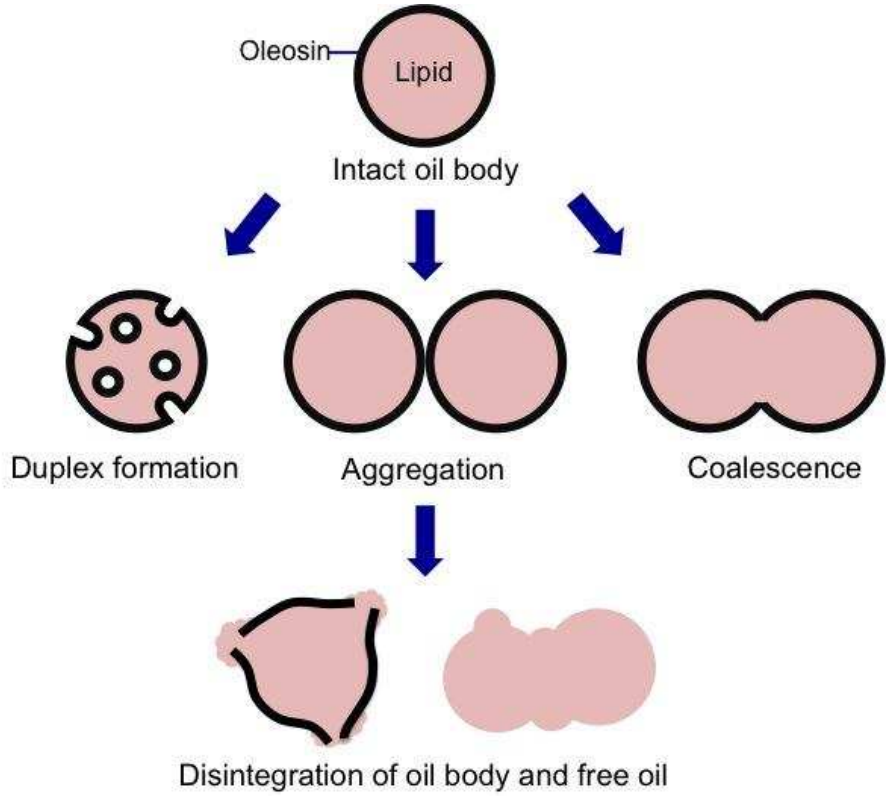


Figure 6.1 Disintegration of oil bodies

Although the levels of unsaturated FFAs in oil bodies (oleic, linoleic and linolenic acids) that have been oxidized to lipid hydroperoxide during storage were not measured, the conversion of unsaturated FFAs to lipid hydroperoxide can be calculated (see Table 6.4). The calculation shows that only small percentages of the unsaturated FFAs were converted to lipid hydroperoxide. During rice bran storage, lipid hydrolysis increased dramatically, but the lipid hydroperoxide levels increased only slightly indicating that the oxidation was relatively low or the levels of lipid hydroperoxides were underestimated by the measurements. Lipid hydroperoxides might

rapidly decompose to other metabolites, which were not determined in this study. In addition, the lipid oxidation in rice bran might be retarded by antioxidant compounds present in rice bran. Tocochromanols and oryzanol can increase oxidative stability of lipids through increasing the induction period by scavenging lipid peroxyl radicals (Frankel, 2005; Juliano *et al*., 2005). In this study, these phytochemicals were shown to be associated with rice bran oil bodies. Since tocochromanols and oryzanol are amphiphilic, they would be expected to be located on the surface of the oil body where oxidation would initially occur (the oil/water interface). The location of these phytochemicals suggests a protective role against oxidation *in vivo*.

Table 6.4 Conversion of unsaturated free fatty acids to lipid hydroperoxides during rice bran storage [1]

Rice bran storage	% Total FFA in total lipids [2]	% Unsat. FFA in total FFA [3]	% LOOH in Unsat. FFA [4]	% Conversion of Unsat. FFA to LOOH [5]
Day0	17	14	0.10	0.71
Day10 25℃	23	19	0.17	0.86
Day10 45℃	48	42	0.31	0.75
Day20 25℃	30	26	0.21	0.81
Day30 25℃	64	57	0.16	0.29
Day40 25℃	ND	ND	ND	ND

1 ND = Not determined

2 % Total free fatty acids
= % oleic acid (g) in total lipid pool (g) x 100

3 % Unsaturated free fatty acid in total FFA pool
= % unsaturated fatty acids in total lipid pool / % FFA x 100

4 % Lipid hydroperoxide in unsaturated free fatty acids
= hydroperoxide (mol/g lipid) x molecular weight of cumene hydroperoxide (152 g/mol) x 100%

5 % Conversion of unsaturated free fatty acid to lipid hydroperoxide
= lipid hydroperoxide in unsaturated free fatty acid pool / unsaturated fatty acid in total FFA pool

It is very interesting that an oil body preparation contained less FFA than the bulk oil from the same bran. Since unsaturated fatty acids can rapidly oxidize, the oxidation may occur faster in the bulk oil than the oil bodies. It is likely that oil bodies can protect the stored lipids against hydrolysis and oxidation (Fisk *et al*., 2008). Oleosins on the surface of oil bodies may act as a physical barrier by shielding the oil body TAG core and prevent it coming in contact with lipase. Antioxidants that are strongly associated with the oil bodies (tocochromanols and oryzanols) may retard lipid oxidation by behaving as a chain-breaking electron donor (Frankel, 2005) and could have contributed to oxidative stability of the oil bodies over the bulk oil.

In contrast, protein may initiate lipid oxidation through the chelation of free metal ions and the formation of peroxyl radicals and reactive carbonyls. A massive decline in extraneous protein bands (SDS-PAGE) and a remarkable decline in FFA (TLC) in the alkali-WOB may suggest that the washed oil bodies would be more stable against oxidation than the oil body preparations. Alkali-WOB may be separated from free oil in the “off” bran, and we might expect a decline in lipid hydroperoxide after washing oil bodies from the stored rice bran with alkaline solution.

By using FFA as a marker for lipid deterioration, viable oil bodies (intact oil bodies, and free of FFAs) can be recovered from rice bran that has gone off. There are two possible reasons for the lower level of FFA in oil bodies than the bulk oil. First, concentration of free oil substrate for lipase is low in the oil bodies. Oil bodies appear to be less efficient substrate than bulk oil since smaller lipid/lipase interface is offered by the oil bodies. Secondly, efficiency of lipase in the oil bodies is low because EDTA in the oil body extraction medium chelates free metal ions (Ca^{2+}) from the oil bodies and thus, deactivates the lipase activities (Hu *et al.*, 2010).

Furthermore, a comparable quantity of viable oil bodies was recovered from fresh and “off” bran (10 days). This indicates that the oil bodies may delay the onset of lipid oxidation of stored lipids inside the oil bodies, and therefore, may reduce the cost required for rice bran stabilization in the rice bran oil industries.

The precise location of hydrolytic and oxidative rancidity in rice bran is not clear from the literature. In this study, FFA and lipid hydroperoxide data suggests that FFAs are produced from TAG associated with oil bodies and that FFA remains unreacted (unless lipid hydroperoxide migrate to the same place on TLC), therefore only small fractions of lipid/fatty acyl groups/FFAs are oxidized. The

lipid hydrolysis and oxidation in rice bran are proposed to come from 3 sources:

1. Free oil in rice bran that can be easily accessed by lipase from the testa-cross layer of the rice kernel and cause an increase of free fatty acids immediately after rice milling.
2. Cellular lipids (e.g. phospholipids of membranes and those surround oil bodies) associated with oil bodies.
3. Oil body TAG that is metabolized during storage as seen by prominent FFA spot and reduced TAG spot on the TLC plate.

One major challenge in this project was to develop a treatment for bran that prevents rancidity but also retains oil body integrity and phytochemical concentration. Traditionally, bran is heat-treated to slow down hydrolytic and oxidative rancidity, but certain time and temperature regimes cause oil bodies to coalesce. Extrusion under conditions required to disable enzymes also leads to wide-spread oil body coalescence. This suggests that the heat stabilization process promotes instability/breakdown of the oil body in rice bran, which limits the oil body recovery. Therefore, enzyme inactivation and the retention of the oil bodies need to be optimized. Alternatively, the stabilization of rice bran may not be required as viable oil bodies can

be recovered from rancid rice bran, but the yield of the oil bodies per unit mass of bran would be less than from fresh rice bran.

6.2. Conclusions

- Brown rice (aged more than one year old) was milled on site and produced fresh rice bran as a by-product for this study.
- Oil bodies were compressed within cells, but remained as small individual organelles (0.5 - 1 μm in diameter) *in vivo* and were enriched in the aleurone and sub-aleurone layers of brown rice.
- Oil bodies in the heat-treated rice bran coalesced and lost surface integrity *in vivo*.
- Oil bodies were successfully recovered from rice bran (fresh and rancid) and could be resuspended to prepare aqueous suspension/emulsion.
- The effective lipid concentration increased while protein concentration decreased after washing the oil bodies with both water-based and alkali-based method.
- All washing protocols did not seem to remove oleosin, and much of the tocochromanols and oryzanol remained; this suggests an intrinsic association between them and rice bran oil bodies *ex vivo*, and, by implication, *in vivo* within the bran of the rice seed.

- Rice bran oil bodies possess a negatively charged surface at neutral pH (-30 mV). As the pH of the oil body suspension is lowered to the pH near pI (about pH 4 - 5), zeta potential of the oil bodies approaches zero and the suspension has the least physical stability leading to more rapid aggregation.
- Increasing temperature and relative humidity during rice bran storage increased the rate of lipid hydrolysis (hydrolysis of TAGs to FFAs) and oxidative rancidity (changes of lipid hydroperoxide).
- The tocochromanol concentrations of rice bran decreased in the temperature dependent fashion while those of oryzanol were relatively steady during rice bran storage.
- Antioxidant activities provided by the remaining tocochromanol and oryzanol may contribute to the low lipid oxidation in rice bran and oil bodies during rice bran storage.
- The storage of rice bran resulted in the decrease of oil body yields, digestion of oleosin and loss of oil body integrity and stability.
- Oil bodies that could be recovered from the stored rice bran contained high levels of tocochromanols (558 ± 12 mg/kg lipid) and oryzanol (9,356 ± 254 mg/kg lipid).
- The high temperature of stabilization caused oil body fusion and coalescence, which limit the recovery of the oil bodies from the heat-treated rice bran.

6.3. Future studies

- It would be of interest to establish the chemical states (pH, solute concentration etc.) of the cytoplasm in rice bran during oil body biogenesis. This would allow one to design conditions *ex vivo* that promotes oil body stability.
- Analysis of phospholipids in rice bran and rice bran oil bodies during storage would allow a better understanding of the mechanism of oil body degradation in rice bran during storage, and help us to identify which lipids are oxidized.
- Although the oxidative stability of oil bodies in stored rice bran was measured, it would also be of industrial significance to measure the oxidative stability of rice bran oil bodies and their washed preparations *ex vivo* over storage or in a product.
- Develop a treatment for bran that can inactivate enzymes, but also retain oil body integrity and phytochemical concentration. Rice bran stabilization by microwave heating would be of interest.

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